Exosomal Regulation of TGF-β Signalling

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Submitted in total fulfilment of the requirements of the degree of Master of Philosophy

May 2015

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Declaration

This is to certify that:

1) The thesis comprises only my original work towards the MPhil except where indicated.

2) Due acknowledgements have been made in the text to all other materials used.

3) The thesis is less than 50,000 words in length, exclusive of tables, figure legends, and bibliographies.

Lin Liu

Hong-Jian Zhu
(Supervisor)

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Acknowledgements

I would like to express gratitude to my supervisor Dr Hong-Jian Zhu, who offered me the opportunity of MPhil study in Australia. Your guidance, encouragement and patience during the past two years of my study have been invaluable. Thank you for your insightful advice, support and assistance throughout the project and during writing up.

I particularly thank Prof. Richard J. Simpson for his assistance throughout my project. I also thank A/Prof. Heung-Chin Cheng and Sio Seng Lio for their help with ultracentrifugation at Bio21 Institute.

I especially thank our research assistant Josephine Iaria for her exceptional technical assistance throughout my project and the care and support she has given us in the laboratory.

I also thank Pierre Smith for computer and information technology support, Annelie Smith and Kim Ng for constant support in the department. I thank Kaixin Zhou for critical reading of the thesis.

I express my special thanks to all members in the cancer signalling laboratory who have made my experience in the past two years an extremely rewarding and enjoyable one.

My sincere thanks are due to my family and friends who have been my constant source of support and advice. Finally but most importantly, I owe my deepest gratitude to my parents, who have given me enormous support for every decision I have made since childhood. Thank you for all the love and care and I hope I have made you proud!

Abstract

Exosomes are nanometer-sized vesicles (40-100 nm) secreted by various cells types. They are generated from late endosomes/multivesicular bodies (MVBs) and are released into extracellular space upon the fusion of MVBs outer membrane with plasma membrane. Exosomes exert a broad range of biological effects by transferring different contents (such as DNA, RNA, proteins and lipids). In particular, multiple signalling molecules (such as Wnt proteins) are carried in exosomes which promote tumor aggressiveness.

Transforming growth factor-β (TGF-β) is a key player in various biological processes. TGF-β signalling is tightly regulated by multiple molecules at each step of the signalling cascade. Dysregulation of TGF-β signalling has been implicated in the pathogenesis of human diseases, including cancer. TGF-β can signal not only via linear signalling cascade but also through crosstalk with other pathways. Importantly, TGF-β and Ras signallings are found to act in synergy to promote malignant progression. Ras enhances TGF-β signalling by suppressing the SPRY domain and SOCS Box containing protein 1 (SPSB1), a negative regulator of TGF-β type II receptor (TβRII). However, the underlying molecular mechanism is controversial because Ras does not change the poly-ubiquitination levels of SPSB1.

Active TGF-β has been discovered in exosomes. Exosomal TGF-β is shown to activate Smad-dependent signalling in recipient cells and drive fibroblast to myofibroblast differentiation. However, it is not clear how exosomes are involved in the regulation of TGF-β signalling. In particular, how exosomal TGF-β may differ from free ligand TGF-β in exerting their biological functions is yet to be answered. Moreover, SPSB1 is detectable in exosomes from Ras-transformed cells, raising the possibility that exosomes participate in Ras-induced up-regulation of TGF-β signalling by exporting SPSB1 from the cell.

This thesis demonstrated that highly metastatic tumor cell lines secreted higher levels of exosomal TGF-β in comparison with non-/low- metastatic tumor cell lines, suggesting the potential of exosomal TGF-β as a biomarker for metastatic cancer. Moreover, exosomal TGF-β activated TGF-β signalling in TGF-β receptor-defective cells. This observation will cause a re-evaluation of TGF-β signalling's role in tumorigenesis of human colon cancer harbouring TβRII inactivating mutation. Importantly, ligand traps were inefficient in blocking exosomal TGF-β-mediated TGF-β signalling. Consequently, strategies have to be developed to target exosomal TGF-β.

Next, the intracellular TβRII negative modulator SPSB1 was discharged by exosomes upon Ras overexpression, resulting in a reduction in the cellular pool of SPSB1. Treatments using small molecular inhibitors indicated that Ras-mediated SPSB1's MVB sorting and exosome secretion were dependent on Ca^{2+} concentration, ceramide and PI3K. SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain was required for SPSB1's co-localization with CD63-positive late endosome and the subsequent release on exosomes. However, exosomal discharge of SPSB1 was independent of the ubiquitination of SOCS box. Furthermore, the SPSB1- and Ras- containing exosomes could be internalized by recipient cells. Collectively, these results highlighted the role of exosomes as vehicles for intracellular protein discharge.

Lastly, this thesis revealed that Ras expression resulted in elevated levels of exosomal TGF-β, implying that Ras acts as a pivotal participator in the production of exosomal TGF-β.

Abbreviations

BMP	Bone morphogenetic protein
CAF	Carcinoma/cancer-associated fibroblasts
DMA	Dimethyl amiloride
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal–regulated kinase
ESCRT	Endosomal sorting complex required for transport
FCS	Fetal calf serum
GAP	GTPase-activating protein
GEF	Guanine exchange factor
GDP	Guanosine diphosphate
GDF	Growth and differentiation factors
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HGF	Hepatocyte growth factor
HIF1α	Hypoxia-inducible factor-1α
ILV	Intraluminal vesicle
kDa	kilo Dalton
LAP	TGF-β latency associated protein
LLC	Large latent TGF-β complex
LMP1	Latent membrane protein 1
LTBP	Latent TGFβ-binding protein
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MMP	Matrix metalloproteinases
MVB	Multivesicular body
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol (PI) 3-kinase
PTEN	Phosphatase and tensin homologue
Ras	Rat sarcoma virus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLC	SLC small latent TGF-β complex
Smad	Sma and Mad related protein
SOCS	Suppressor of cytokine signalling
SPRY	Sequence repeat in the dual-specificity kinase splA and ryanodine
SPSB1	SPRY domain and SOCS Box containing protein 1
sTβRII-Fc	Soluble TGF-β receptor II-Fc
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-β
TβRI	Transforming growth factor-β type I receptor
TβRII	Transforming growth factor-β type II receptor
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

Chapter 1. Literature Review

Human's knowledge of "cell" dates back to 1665, when Robert Hooke first discovered cells in cork. In 1839, Theodor Schwann and Matthias Jakob Schleiden introduced the "cell theory", in which they elucidated that all organisms were composed of cells [1]. With the development of modern microscopes in 20th century, architecture of eukaryotic cells were revealed and confirmed. Eukaryotic cells were then described as a diverse community of organelles with various functions as described in Table 1.1 [2-12].

In 1940, the discovery of cell-derived vesicles shed new light on our understanding of subcellular structures. The clotting-promoting factors in plasma were shown to be small vesicles (20-50nm in diameter) [13]. In 1987, those vesicles were first termed as "exosomes" when Rose M. Johnstone and colleagues isolated sphingomyelin-rich vesicles from the conditioned medium of sheep reticulocyte [14]. These transferrin receptor-containing exosomes had no lysosomal activities, indicating that exosomes might be a major vehicle for the removal of transmembrane proteins [15]. Also, exosomes secreted by Epstein-Barr virus-transformed B-lymphocytes were shown to stimulate T cell proliferation, revealing their biological role as mediators of cell-cell communication [16].

Considering their crucial functions, it is exciting to study exosomes, the newest family member of bioactive vesicles. Knowledge and techniques in investigating exosomes are advancing rapidly. Over the last few years, studies have been focused on the biogenesis and secretion of exosomes, the cargoes carried in exosomes and their biological effects on recipient cells.

Table 1.1 Organelles in eukaryotic cells; histories and functions [2-12].

Organelles	History	Function
Cell Nucleus	Discovered by Robert Brown in 1833	Regulating gene expression and DNA replication during cell cycle; controlling cell behaviour
Centrosome	Discovered by Edouard Van Beneden in 1883; described and named by Theodor Boveri in 1888	Mitotic spindle building
Mitochondria	Observed by Albert von Kolliker in 1857; named by Carl Benda in1898	Producing ATP; regulating cellular metabolism
Golgi Apparatus	Identified by Camillo Golgi in 1897	Modifying, sorting and packaging macromolecules
Endoplasmic Reticulum	Discovered by Albert Claude and Keith Porter in 1945	Folding and transporting synthesized protein to Golgi apparatus
Lysosomes	Discovered by Christian de Duve in 1950s	Digesting waste materials and cellular debris
Ribosomes	Discovered by George Palade in 1955	Translating mRNA into protein
Peroxisomes	Described by J. Rhodin in 1954; identified by Christian de Duve in 1967	Ridding toxic peroxides from cells

1.1 Exosomes: biogenesis, secretion and cargoes

Exosomes are small membrane vesicles (40-100nm in diameter) secreted by various cell types [17]. They are generated from the inward budding of the endosome's limiting membrane into its lumen, forming multivesicular body (MVB) [18]. Upon the fusion of MVB outer membrane with plasma membrane, their intraluminal vesicles (ILVs) are released to the extracellular space as exosomes [19] (Fig. 1.1). Because of their MVB origin, exosomes contain proteins and lipids that participate in membrane trafficking and MVB biogenesis. For instance, the endosome membrane protein Tsg101 is defined as an exosome marker. Alix, a cytosolic protein that regulates endosomal trafficking [20], is also found in the exosome proteome [21]. Additionally, studies have shown enrichment of tetraspanin family members such as CD 37, CD63 and CD81 in MVBs and exosomes [22].

1.1.1Exosome Biogenesis and Secretion

(1) Exosome biogenesis

As mentioned above, exosomes originate from MVBs. The biogenesis of exosomes can be described as an orderly sequence of basic steps. First, cargoes are segregated and recruited within the limiting membrane of the late endosome. Then, the inward-budding vesicles form and pinch into endosome lumen, forming MVB. Finally, MVB fuses with plasma membrane, releasing its intraluminal vesicles into extracellular space as exosomes.

The endosomal sorting complex required for transport (ESCRT) machinery has been identified to recognize and sequester ubiquitinated proteins for MVB sorting [23, 24]. In this case, ubiquitination of cargoes serves as a sorting signal [25]. Studies suggest that ESCRT-0, through its ubiquitin-binding motif, recognizes and engages ubiquitinated proteins, while ESCRT-I and ESCRT-II initiate the membrane budding. After the recruitment of Alix or Nedd4 family proteins [21], ESCRT-III mediates the pinch off of the cargo-containing vesicles into endosome lumen, forming the ILVs of MVBs [52]. The ESCRT-0 component HGS has been demonstrated to take part in Evi-Wnt3a secretion on exosomes [26]. In yeast cells, the mono-ubiquitination of Rim8, an arrestin-related protein, was found to facilitate its association with ESCRT-I subunit Vps23 [53]. Similar study in mammalian cells revealed that the epidermal growth factor receptor (EGFR) was a target of ubiquitin ligase UBE4B during endosomal sorting [27].

Apart from ESCRT-mediated endosome sorting, ceramide-dependent mechanism has been reported to regulate exosome release [28]. Ceramide is produced upon the removal of phosphocholine from sphingomyelin (SM) by sphingomyelinases (SMases) [29]. Because of the structural properties, SMase treatment or addition of C6-ceramide causes vesicles formation [30-32]. It has been indicated that purified exosomes are enriched in ceramide. After treatment with the neutral sphingomyelinase (nSMase) inhibitor, GW4869, the exosome secretion in Oli-neu cells was significantly reduced [26, 28]. Also, GW4869 blocked the exosomal discharge of β-catenin, which was mediated by tetraspannins CD9 and CD82 [18]. Collectively, these data demonstrate the role of ceramide in exosome biogenesis and release.

Additionally, studies have provided evidence that lipid metabolism is involved in MVBs formation and exosome biogenesis. In particular, yeast and mammalian cells require phosphatidylinositol (PI) 3-kinase (PI3K) [33] as well as PI(3)-phosphate 5 kinase activities [34] in their MVB pathway. PI3K has been shown to localize to plasma membrane, intracellular vesicles, as well as cytoplasmic lipid bodies [35]. Of note, PI3K can be recruited to endosome by Ras, and exert their signalling activity in this compartment [36]. The invagination and pinching off of intraluminal vesicles were blocked by PI3K inhibitor, wortmannin. As a result, vacuolation of early and late endosomes [37] as well as decreased exosome secretion were observed [38], indicating the role of PI3-kinase in MVB morphogenesis.

Moreover, tetraspannins have been implicated in exosome biogenesis/secretion. The tetraspannin superfamily is defined to contain four transmembrane domains and have a broad subcellular distribution, including cell surface, endosomal system and lysosome. Among tetraspannins, CD37, CD63, CD81 and CD82 are abundant on exosomes [22]. In particular, CD63 are predominantly localized to late endosomes/MVBs and lysosomes, and are usually referred to as markers of late endosomes/MVBs and lysosomes [39]. CD63 appears to associate with latent membrane protein 1 (LMP1) and mediate their exosomal export, hence attenuating the constitutive activation of NF-κB [40]. Also, CD9 and CD82 have been linked to the exosomal release of β-catenin, which in turn contributes to the inhibition of Wnt signalling [18].

(2) Regulation of exosome secretion

It is well documented that Rab family of small GTPases control intracellular trafficking. To date, 70 Rab proteins have been identified in human. They show different subcellular distributions and are involved in regulating various vesicular trafficking processes, including budding, docking, vesicle fusion and transport [41, 42]. Some of the Rab proteins have been identified to play crucial roles in exosome production and secretion. For example, Rab5 regulates early endosome biogenesis. The GTP-locked mutant form of Rab5 (Rab5Q79L) enlarged the early endosomes and therefore blocked the release of exosomes [21, 28]. Rab11 was found to work in collaboration with Syntaxin 1A (Syx1A) in modulating the secretion of exosomes containing Evenness Interrupted (Evi), a Wg-binding protein [43]. Both Rab35 and its upstream GTPase-activating proteins (GAPs) TBC1D10A were required for exosome release in neuron cells via influencing MVBs docking and tethering [44]. Notably, a number of studies have uncovered the involvement of Rab27a/b in exosome secretion [45]. A RNA interference screen targeting human Rab genes identified five Rab proteins (Rab2b, Rab9a, Rab5a, Rab27a and Rab27b) which were responsible for exosome release. Among them, Rab27b were shown to mediate the transfer of membranes from the trans Golgi network (TGN) to MVBs, while Rab27a affected vesicles docking and fusing with the plasma membrane [41]. Another study suggested that Rab27a/b participated in exosomal discharge of cytosolic tumor-suppressive miRNAs. Rab27a/b knockdown in bladder cancer cells led to an intracellular enrichment of tumor-suppressive mRNAs, coinciding with a decreased cell invasion [46]. Furthermore, Rab27a has been linked to tumor metastasis in melanoma via promoting exosome production and the release of proangiogenic factors [47].

Also relevant to exosome secretion is the intracellular calcium changes [48, 49]. During exocytosis, the fusion of secretory vesicles with plasma membrane is tightly controlled by Ca^{2+} concentration. After treatment with Monensin, a membrane permeable Na^{+}/H^{+} exchanger which is able to induce changes in intracellular calcium concentration, larger MVBs as well as enhanced exosome release were observed [38, 50]. Conversely, exosome secretion was blocked by dimethyl amiloride (DMA), an inhibitor of Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchanger [51].

Additionally, many proteins have been described to take part in regulating exosome production. Study showed that exosome release was enhanced after DNA damage. This response was mediated by p53 target gene, TSAP6. Thus, p53 responded to stress via up-

regulating the production of exosomes, which might facilitate the activation of immune system [52]. The exosomal sorting and secretion of ubiquitin ligase Nedd4 family were found to depend on the expression of adaptor protein, Nedd4 family-interacting protein 1(Ndfip1) [53]. Syndecan-4/syntenin pathway was involved in the secretion of exosomal Angiopoietin-2 by endothelial cells [54]. Also, VAMP7 and ATPase NSF have been demonstrated to control the fusion of MVB with plasma membrane, and the subsequent release of ILVs as exosomes [55].

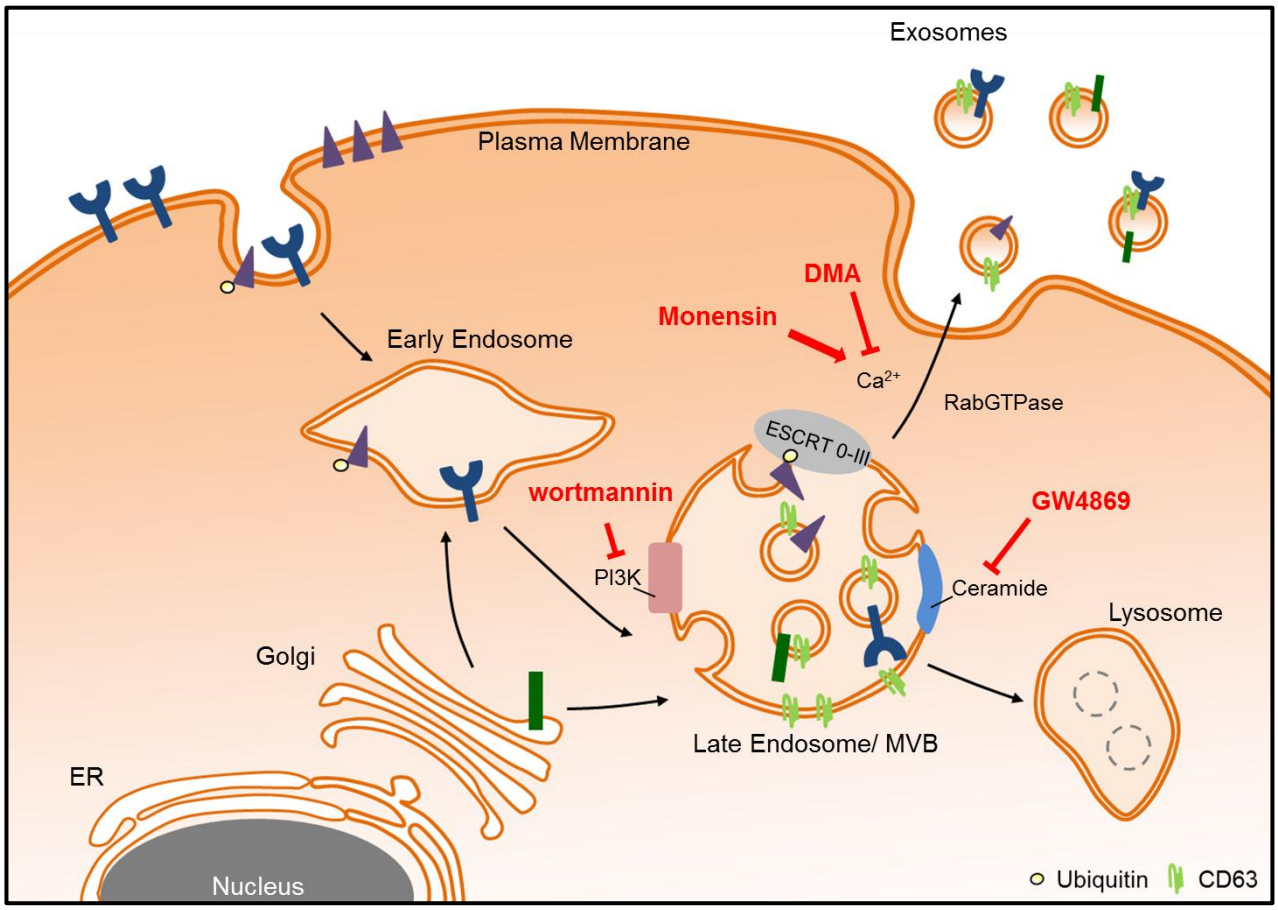


Figure 1.1. Exosome biogenesis and secretion. Exosomes are generated from the inward budding of the endosome's limiting membrane into its lumen, forming multivesicular bodies (MVBs). Upon the fusion of MVBs outer membrane with the plasma membrane, their intraluminal vesicles (ILVs) are released to the extracellular space as exosomes. It has been shown that endosomal sorting complex required for transport (ESCRT) machinery, ceramide and PI3K are involved in exosome biogenesis. The ESCRT machinery has been identified to recognize and sequester ubiquitinated proteins for MVB sorting. PI3K-mediated and ceramide-dependent MVB sorting can be inhibited by small molecular inhibitors, wortmannin and GW4869, respectively. Exosome release is tightly controlled by RabGTPase and Ca^{2+} concentration. Ca^{2+}-dependent exosome secretion is inhibited by DMA, the inhibitor of Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchanger while enhanced by Monensin, a membrane permeable Na^{+}/H^{+} exchanger.

1.1.2 Exosomal cargoes

As mediators of intercellular communication, exosomes carry a broad range of cargoes (such as proteins, genetic materials, and lipids) (Fig. 1.2).

(1) Genetic materials

A well-known function of exosomes is to shuttle genetic materials, including DNAs, message RNAs (mRNAs) and microRNAs (miRNAs). Apart from mitochondrial DNA (mtDNA) and single-stranded DNA (ssDNA) [56, 57], double-stranded DNA (dsDNA) has also been found in exosomes. A recent study showed that dsDNAs of mutated KRAS and p53, the most frequent mutations in human pancreatic cancer, were in the exosomes isolated from pancreatic cancer cell lines. Importantly, exosomal DNAs contained the same mutation as the parental cell line, such as *BRAF (V600E)* and *EGFR*, suggesting the potential of exosomal DNAs as attractive biomarkers in cancer detection [58].

Microarray analysis of exosomal RNAs revealed that mRNAs, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) were secreted in association with exosomes [59, 60]. While the average size of mammalian mRNAs is 2100nt [61], the mRNAs carried in exosomes are smaller (25-700nt) [62, 63]. The mRNAs encoding tRNA synthesis and prostate cancer-associated splice variant have been discovered in exosomes [64]. TGF-β1 mRNA encapsulated in exosomes from injured epithelial cells was able to initiate tissue regenerative response. More importantly, exosome mRNA profiling differentiates between healthy and cancer patients [65]. For instance, the mRNA for EGFRvIII has been found in exosomes derived from glioblastoma patient, but not in normal samples [66]. A growing body of evidence implicate the role of exosomal mRNA in tumor progression. Hong et al. analysed the transcriptome of exosomes released from SW480, a human colorectal cancer cell line. They found the enrichment of cell cycle-related mRNAs which could stimulate the endothelial cell proliferation [67]. Exosomal ΔNp73 mRNA derived from tumor cells promoted tumorigenic process by benefiting proliferation and chemoresistance of recipient cells [68]. Also, mRNA transcripts in glioblastoma exosomes have been linked to tumor malignancy such as cell proliferation, migration and angiogenesis [66].

The discovery of miRNAs in exosomes is the most significant advancement in exosome biology [69]. miRNAs are short non-coding RNAs that target mRNAs or affect protein translation, mediating gene down-expression at post-transcriptional level. It has been found

that the loading of miRNAs into exosomes was controlled by their EXOmotif and the sumoylation by protein hnRNPA2B1[70]. As such, some miRNAs were specifically observed in exosomes while the others retained in the parental cells [70]. In particular, a recent study showed the exosomal secretion of miRNAs with tumor-suppressive function (such as *miR-145, miR-23b, miR-921, miR-224*) by metastatic bladder cancer cells, which might support the metastatic behaviour of the tumor cells [46]. Similarly, the enrichment of *let-7* miRNA family in exosomes correlated directly with the metastatic property of gastric cancer cell line [71]. Also, exosomes contained *lin-4* miRNA and *miR-375,* which were involved in development timing and exocytosis, respectively [59]. Taken together, these studies suggest the multifunctional roles of exosomal miRNAs.

LncRNAs are non-protein coding transcripts longer than 200nt. In recent years, lncRNAs are gaining increasing interest as key players in multiple cellular contexts [72]. The discovery of exosomal lncRNAs highlights their role in mediating cell-cell communication. For example, exosomes from both MCF-7 cells and HeLa cells were shown to contain high levels of *lncRNA-p21,* a repressor of *p53*-mediated gene expression [60]. Moreover, exosomal *lncRNA-ROR* has been implicated in modulating cell viability after chemotherapy [73].

(2) Tumor-promoting proteins

Exosomes have been shown to play a pivotal role in manipulating tumor microenvironment by horizontal delivery of tumor-promoting proteins. The proteome profiling of exosomes derived from two human colorectal cancer cell lines, SW480 (primary) and SW620 (metastasis), revealed the presence of K-Ras, N-Ras and H-Ras on exosomes [74]. In particular, Ras were heavily enriched in exosomes from the highly metastatic SW620 cells. Importantly, the delivery of mutant K-Ras via exosomes resulted in enhanced three-dimensional growth of non-transformed cells [75].

Recent reports also suggested the presence of oncogenic EGFRvIII in the microvesicles shed from aggressive human brain tumors. Skog et al. showed that EGFRvIII were transported by membrane-derived microvesicles (termed as oncosomes at that time) between glioma cells. The uptake of EGFRvIII-exosomes by EGFRvIII-negative recipient cells resulted in the activation of transforming signalling pathways as well as the expression of EGFRvIII targeting genes such as VEGF and p27, and the subsequent promotion of anchorage-independent growth of tumor cells [76]. In another study, EGFRvIII was identified in the

brain tumor exosomes by proteomic analysis. These brain tumor-derived exosomes participated in immune modulation [77].

Moreover, the exosomal secretion of oncogenic LMP1 has been observed [40]. LMP1 was found to drive constitutive NF-kB activation and epithelial-mesenchymal transition (EMT). LMP1's sorting into MVBs and exosomes occurred through associating with the tetraspannin CD63. The uptake of LMP1-containing exosomes resulted in the activation of PI3K and ERK signalling in recipient cells [78]. Notably, hypoxia-inducible factor1α (HIF1α), an oncogenic transcription factor which is induced by LMP1 expression [79], was also recruited to exosomes by LMP1. The levels of active exosomal HIF1α have been linked to EMT progression and the invasive potential of recipient cells [80].

A recent study by Atay et al. revealed a relationship between oncogenic protein tyrosine kinase (KIT)-containing exosomes and the gastrointestinal stromal tumor development [81]. Uptake of exosomal KIT by acceptor cells resulted in enhanced invasiveness via activating KIT downstream factors, particularly matrix metalloproteinases 1 (MMP1). This study suggested a new anti-cancer strategy by targeting exosomal KIT.

(3) Tumor suppressor proteins

Apart from oncogenic cargoes, tumor suppressor has also been discovered in exosomes. For example, Putz et al. observed an enrichment of phosphatase and tensin homologue (PTEN), a tumor suppressor protein mainly localized in cytosol and nucleus, in exosomes [82]. Specifically, both the ubiquitination of PTEN as well as the adaptor protein Ndfip1 were required for PTEN's exosomal packaging. Moreover, PTEN-containing exosomes inhibited the proliferation of recipient cells by suppressing PI3K/AKT signalling [82]. While exosomal PTEN has been revealed, other exosomal tumor suppressors remain to be identified.

(4) Ligands and Receptors

Exosomes have been implicated in intercellular communication by transferring signalling molecules. Signalling cargoes are encapsulated into exosomes to ensure traffic specificity, and also the stability of the cargoes. For example, the lipid-modified ligand Wnt3a was shown to be carried on exosome surface, thus exerting their patterning decision function over a distance [26]. Also, Wnt11 produced by breast cancer cells (BCCs) were tethered on fibroblast-derived exosomes, which in turn enhanced the invasiveness of BCCs through Wnt-planar cell polarity (PCP) signalling [83]. The Notch signalling ligand, Delta-like 4 (DII4),

was incorporated into exosomes [84] to influence angiogenesis [85] beyond direct cell-cell contact. Moreover, exosomes from fibroblasts obtained from individuals with rheumatoid arthritis (RASF) were characterized to express tumor necrosis factor-α (TNF-α) and participate in apoptosis resistance [86].

The exosomal secretion of epidermal growth factor (EGF) ligands and receptors is well documented. For example, EGFR ligands (such as TGF-α and amphiregulin) were detectable in exosomes derived from multiple cell lines. In particular, the amphiregulin (AREG)-containing exosomes activated the EGFR in recipient cells and greatly promoted the invasiveness of recipient cells [87]. As mentioned earlier, oncogenic EGFRvIII, the truncated form of EGFR was carried on exosomes and promoted cell proliferation via activating MAPK/ERK signalling pathway in recipient cells. Studies also reported that exosomes were enriched in full-length EGFRs [88], such as ErbB1 [89]. More importantly, exosomal packaging of ErbB1 was enhanced upon EGF stimulation, suggesting the involvement of ligand-dependent ErbB1 activation in the exosomal incorporation of ErbB1. Also, hepatocyte growth factor (HGF) ligands [90] and receptors (such as MET) [47] were carried in exosomes and promoted tumor progression.

Strikingly, by examining the immunosuppressive tumor exosomes, Jason et al first demonstrated the expression of active transforming growth factor-β (TGF-β) by cancer-derived exosomes [91]. In 2010, the same group revealed the differential production of TGF-β-positive exosomes by multiple cancer cell lines. Also, their study indicated that active TGF-β were tethered on the exosome surface by transmembrane proteoglycan betaglycan (also known as TGF-β type III receptor [92]), and were able to activate Smad-dependent signalling in recipient cells [93]. However, it is not clear whether exosomes also contain TGF-β type I receptors (TβRIs) and TGF-β type II receptors (TβRIIs). It has been observed that TGF-β ligand-receptor complexes undergo endocytosis and are targeted to endosomal trafficking [94, 95], where exosomes are generated. Thus, it is likely that TβRI and TβRII are released on exosomes. In this thesis, we aim to investigate the molecular nature of TGF-β-positive exosomes and to explore their biological functions.

(5) Proteinases and molecular chaperones

Exosomes have been shown to modify the extracellular matrix (ECM) via secreting proteinase. For example, MT1-MMP (MMP-14) [96] and ADAMs [97] were detected in tumor-derived exosomes. Notably, proteinases carried in exosomes are functionally active in degrading ECM components, such as collagen. The exosome enrichment of proteinases has been linked to the invasiveness of tumor cells. A proteomic study revealed an increased abundance of matrix metalloproteinases (MMP-1, MMP-14 and MMP-19) in exosomes released from 21D1 cells (Ras-transformed MDCK cells) as compared with exosomes from MDCK cells [98]. Moreover, some MMP activator proteins (such as molecular chaperones the heat shock protein 90 and 70 (Hsp90 and Hsp70)) have been found in the exosomes. Exosomal Hsp90 was shown to enhance ECM remodelling via activating MMP-2 [99] while exosomal Hsp70 was identified to promote cell invasion [100]. Overall, these studies suggest a correlation between exosome-associated proteinases (and their activators) and the invasiveness of tumors [101].

(6) Lipids

As small vesicles contain lipid bilayers, exosomes carry lipidic molecules. Some lipids are involved in exosome biogenesis, while the others are bioactive and mediate cell-cell communication. Exosomes are enriched in (1) polyglycerophospholipid such as bis(monoacylglycero)phosphate (BMP); (2) phosphatidic acid (PA); (3) sphingolipid such as ceramide; (4) raft-associated lipids such as cholesterol; (5) glycerophospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). In particular, PS was responsible for the rigidity and stability of exosomes [102-104] while PA participated in membrane fusion. Moreover, exosomes transported prostaglandin E2 (PGE2) which had been shown to suppress immune response [105]. The lipolytic enzyme such as phospholipases A2 (PLA2) has also been found to associate with exosomes [104].

(7) Others

Other molecules commonly secreted in exosomes are tetraspannin family (such as CD9, CD63, CD81 and CD82), cytoskeletal proteins (such as tubulin and actin), flotillin and RabGTPase family. Because of their MVB origin, exosomes also contain proteins that are

involved in MVB biogenesis (such as Alix and TSG101) [102]. Consequently, these proteins are usually defined as typical markers of exosomes.

Notably, a recent study revealed the existence of different exosome subpopulations [106]. Through immunoaffinity capture, two subtypes of exosomes (A33-positive and EpCAM positive) were isolated from human colorectal cancer cell line LIM1863. While EpCAM-positive exosomes were enriched in apical sorting proteins, A33-postive exosomes showed an abundance of basolateral sorting proteins. Although the exosome biogenesis pathways have been well documented, this study suggests that different endosomal sorting machineries may exist, resulting in the diversity of exosome composition. Yet, whether exosome subpopulations behave differently in tumor progression remains to be determined.

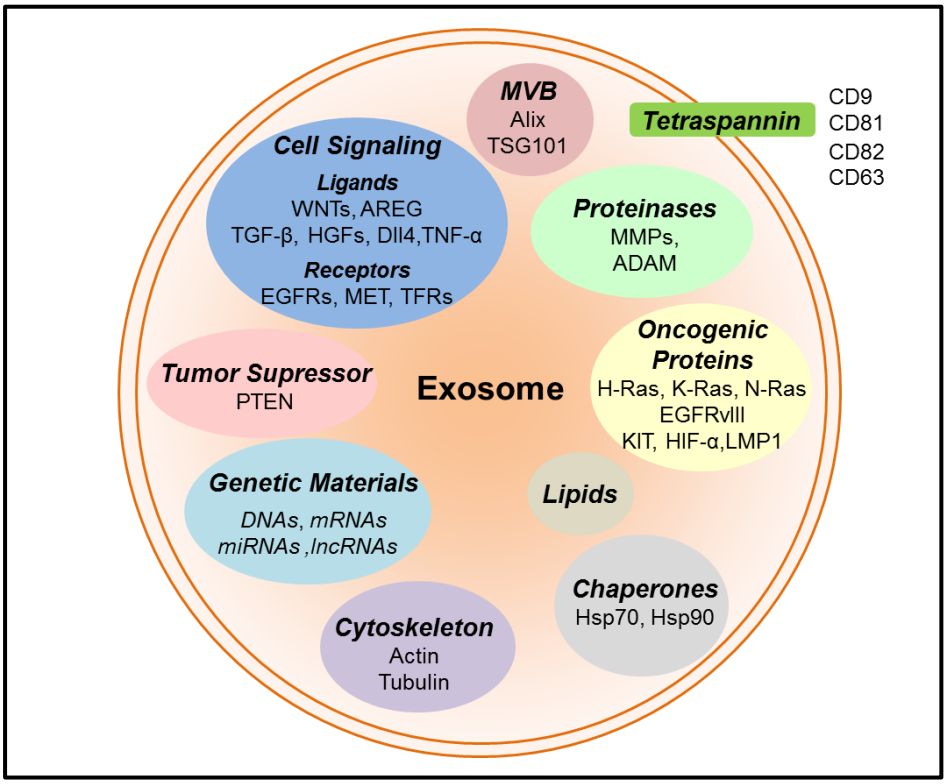


Figure 1.2. Exosomal cargoes. As mediators of intercellular communication, exosomes carry a broad range of cargoes (such as proteins, genetic materials, and lipids).

1.2 Cancer exosomes

In recent years, studies have been focused on the biogenesis and composition of exosomes as well as the regulation of exosome release. Currently, exosomes are increasingly being recognised as key players in human disease, particularly cancer. Exosomes affect tumor progression by regulating immune responses and facilitating tumor formation (promoting cell proliferation, invasion and metastasis, angiogenesis as well as modulating ECM and tumor microenvironment) (Fig. 1.3).

1.2.1 Regulation of immune responses

Tumor-derived exosomes interfere with antitumor immune response. For example, nasopharyngeal carcinoma (NPC) cells have been shown to release exosomes containing galectin-9, which inhibited Th1 cell-mediated immune response by inducing apoptosis [107]. It has also been demonstrated that the expression of T cell signalling components, CD3-ζ and Janus kinase (JAK), were suppressed by FasL-positive exosomes from ovarian cancer patients. Cancer exosomes therefore impaired T cell signalling [108]. Additionally, studies have provided evidence that tumor-derived exosomes blocked the cytotoxicity of natural killer (NK) cells. These exosomes contained molecules that triggered the down-regulation of NKG2D [109]. Specifically, tumor-derived exosomes exert immunosuppressive function through modulating peritumor environment. A recent study by Clayton et al. revealed that cancer exosomes attenuated T cell-mediated immune response by inducing the negative regulator, adenosine. The CD39- and CD73- positive exosomes were found to have ATP-hydrolytic activity, thus resulting in elevated adenosine in extracellular environment and the suppression of T cell signalling [110].

Notably, studies have also demonstrated the anti-tumor responses caused by exosomes. For example, the Hsp70-positive exosomes derived from human pancreatic and colorectal cancer cell lines were shown to enhance the cytotoxicity of NK cells [111]. Given the contradictory immune responses triggered by tumor-derived exosomes, further research is required for targeting exosomes in cancer therapy.

1.2.2 Promotion of cell proliferation

It is well documented that exosomes contribute to tumor progression by promoting cell proliferation. Exosomes derived from gastric cancer cells were capable of up-regulating the PI3K/Akt and MAPK/ERK signalling, thus accelerating the proliferation of recipient cells [112]. Other mechanisms by which the exosomes may affect proliferation have also been revealed. For example, a study by Xiao et al. showed that mast cell exosomes with a high content of oncogenic protein KIT could be delivered to human lung adenocarcinoma cells, thereby triggering increased cyclin D1 expression and enhanced cell proliferation via activating KIT-SCF signalling [113]. Also, some cell cycle-related mRNAs were found to associate with colorectal cancer exosomes. These exosomes could promote cell proliferation when internalized by endothelial cells. Moreover, a recent study revealed an abundance of oncogenic proteins and a lower level of tumor suppressor *miR-15a* on exosomes from multiple myeloma bone marrow mesenchymal stromal cells (MM BM-MSCs) as compared with normal BM-MSCs exosomes. MM tumor growth was promoted by MM BM-MSCs exosomes while suppressed by normal BM-MSCs exosomes [114]. Collectively, these studies suggest the contribution of tumor-derived exosomes to cell proliferation during cancer progression.

1.2.3 Facilitating invasion and metastasis

Metastasis is the major cause of cancer-related death. Cancer cells undergoing EMT acquire the capacity to migrate and invade the surrounding tissue. After travelling in blood and lymphatic vessels, cancer cells extravasate into a distant tissue, seed and grow as secondary tumors (metastasis). It is well documented that tumorigenic molecules secreted by cancer cells are recruited to the potential metastatic site, where the microenvironment is also restructured, forming the pre-metastatic niche [115, 116]. In the last few years, compelling evidences have suggested that exosomes contribute to tumor aggressiveness by regulating different steps of invasion-metastasis cascade.

Exosomes have been found to transport EMT inducers. The HIF1α- and LMP1-positive exosomes could enhance the invasion of recipient cells. The increased invasiveness induced by exosomes was accompanied by the down-regulation of E-cadherin and up-regulation of N-cadherin [80]. Tspan8 and CD151, two metastasis-promoting tetraspanins, could be transported on exosomes to promote the metastasis of ASML-CD151/Tspan8^{kd} cells via driving EMT [117]. Exosomes released from cancer-associated prostate stromal fibroblasts

were enriched in *miR-409*, which could induce EMT and the subsequent tumorigenesis. Also, TGF-β, a key inducer of EMT, was discovered in exosomes [91]. In addition, the reprogramming of exosomal contents following EMT has been observed. For example, induction of mesenchymal phenotype in epithelial A431 cells caused the loss of tissue factors (TF) and EGFR on the cell membrane. However, TFs were shown to be secreted on exosome-like EVs and therefore internalized by endothelial cells [118]. A recent proteomic study on the exosome proteome of MDCK cells and 21D1 cells (Ras-transformed MDCK cells) suggested that exosomes from 21D1cells expressed less epithelial markers (such as E-cadherin and EpCAM) and higher levels of mesenchymal markers (such as vimentin) as well as proteinases. Notably, some of these proteinases have been linked to metastasis niche preparation and tumor microenvironment modification [101, 119]. Moreover, exosome composition was shown to be affected by mutant KRAS status. A higher level of tumor-promoting proteins was found on exosomes derived from mutant KRAS cells as compared with the wild-type KRAS cells [75]. Taken together, these studies indicate that changes in exosome proteome are required for EMT not only in parental cells but also in recipient cells [101].

The crucial role of exosomes in cell invasion and metastasis is well recognized. Singh et al. observed that metastatic breast cancer cells regulated the invasiveness of non-malignant cells through exosomal delivery of *miR-10b.* The internalized *miR-10b* functionally repressed the target genes (such as HOXD10 and KLF4), coinciding with an increased invasiveness of recipient cells [120]. Similarly, bladder carcinoma cells facilitated the acquisition of metastatic property via exosomal transport of miRNAs [46]. Interestingly, these miRNAs (such as *miR-23b*) were shown to inhibit tumor cell invasion and metastasis. This study suggested the role of exosomes as vehicles for discharge of tumor-suppressive miRNAs in the metastatic processes. Apart from miRNAs, exosomal transport of proteins has also been implicated in metastasis. As mentioned above [87], ARGE, the EGFR ligand, is secreted in association with exosomes. Upon uptake, ARGE-positive exosomes enhanced the invasive potential of recipient cells. Moreover, exosomes from mutant KRAS cells displayed higher ARGE levels than the wild-type KRAS cells. Similarly, exosomes secreted by cancer-associated fibroblasts (CAFs) were shown to be internalized by breast cancer cells (BCCs) where they were loaded with BCCs-produced Wnt11 and therefore drove the migration and invasion of BCCs via activating the Wnt-PCP signalling [83]. Also, macrophages could shuttle Wnt5-containing exosomes to breast cancer cells and enhance the invasiveness of

recipient cells [121]. In particular, a recent study has linked exosome secretion to invadopodia formation. Invadopodia facilitated exosome secretion via providing docking site for CD63-positive and Rab27-positive MVBs, while exosomes promoted invadopodia formation and cancer cell invasion. These observations highlighted the existence of a reciprocal loop between exosomes release and invadopodia biogenesis, which might drive the invasion of cancer cells [122].

Exosomes also contribute to pre-metastatic niche formation and tumor microenvironment generation. The melanoma MET receptor-containing exosomes were shown to educate bone marrow progenitors towards a pro-metastatic phenotype and recruite them to pre-metastatic sites, hence supporting tumor metastasis [47]. The TGF-β-positive exosomes derived from cancer cells induced fibroblast to myofibroblast differentiation, a key step in pre-metastatic niche formation [93]. It has also been observed that exosomes cooperated with CD44v, a main actor in soluble matrix assembling, in creating a permissive environment for metastatic tumor cells [123]. Tumor-derived exosomes that were enriched in adhesion molecule (such as CD49c and α6β4) and proteinase (such as MMPs) bound and degraded the extracellular matrix, therefore favoring cell invasion and survival [124]. In addition, melanoma exosomes were found to prepare lymph nodes for metastasis through recruiting pro-tumorigenic factors, remodelling ECM as well as promoting vascularization [125]. This study proposed a mechanism by which cancer exosomes may support lymphatic metastasis.

1.2.4 Inducing angiogenesis

The growth and spread of tumors require the formation of new blood vessels, known as angiogenesis. Various lines of evidence have demonstrated the involvement of exosomes in this process. For instance, the tetraspanin Tspan8 has been shown to associate with tumor-derived exosomes [126]. The Tspan8-positive exosomes were able to recruit selected mRNAs (such as *VEGF* mRNA) and proteins (such as CD49d) which contributed to exosome-endothelial cell (EC) interactions and the subsequent internalization by endothelial cells. Upon uptake, the Tspan8-containing exosomes promoted endothelial cell activation and angiogenesis by regulating angiogenic factor–related pathways. DII4, a Notch signalling ligand, has been found to be transferred between cells via exosomes [84]. The exosomal DII4 could be incorporated into the plasma membrane of recipient cells, where they bound to Notch receptors and caused endocytosis. As such, DII4-containing exosomes down-regulated the Notch signalling, hence inducing angiogenesis. The endothelial cell-derived exosomes

enriched in *miR-214* were shown to elicit their pro-angiogenesis effects through inhibiting senescence of target cells [127]. Exosomes can also influence the angiogenic capacity and the malignancy of melanoma cells in a Wnt5a-dependent manner [128]. It was recently reported that Wnt5a, a non-canonical Wnt ligand, promoted the secretion of exosomes which were comprised of immunosuppressive factors as well as pro-angiogenic molecules, including interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF). Apart from regulating exosomes release, Wnt5a has also been linked to the elevated mRNA levels of endothelial cell-selective adhesion molecule (ESAM), an angiogenesis marker. In addition, a proteomic study demonstrated that some pro-angiogenic and pro-metastatic factors were carried in tumor-derived exosomes under hypoxia [129].

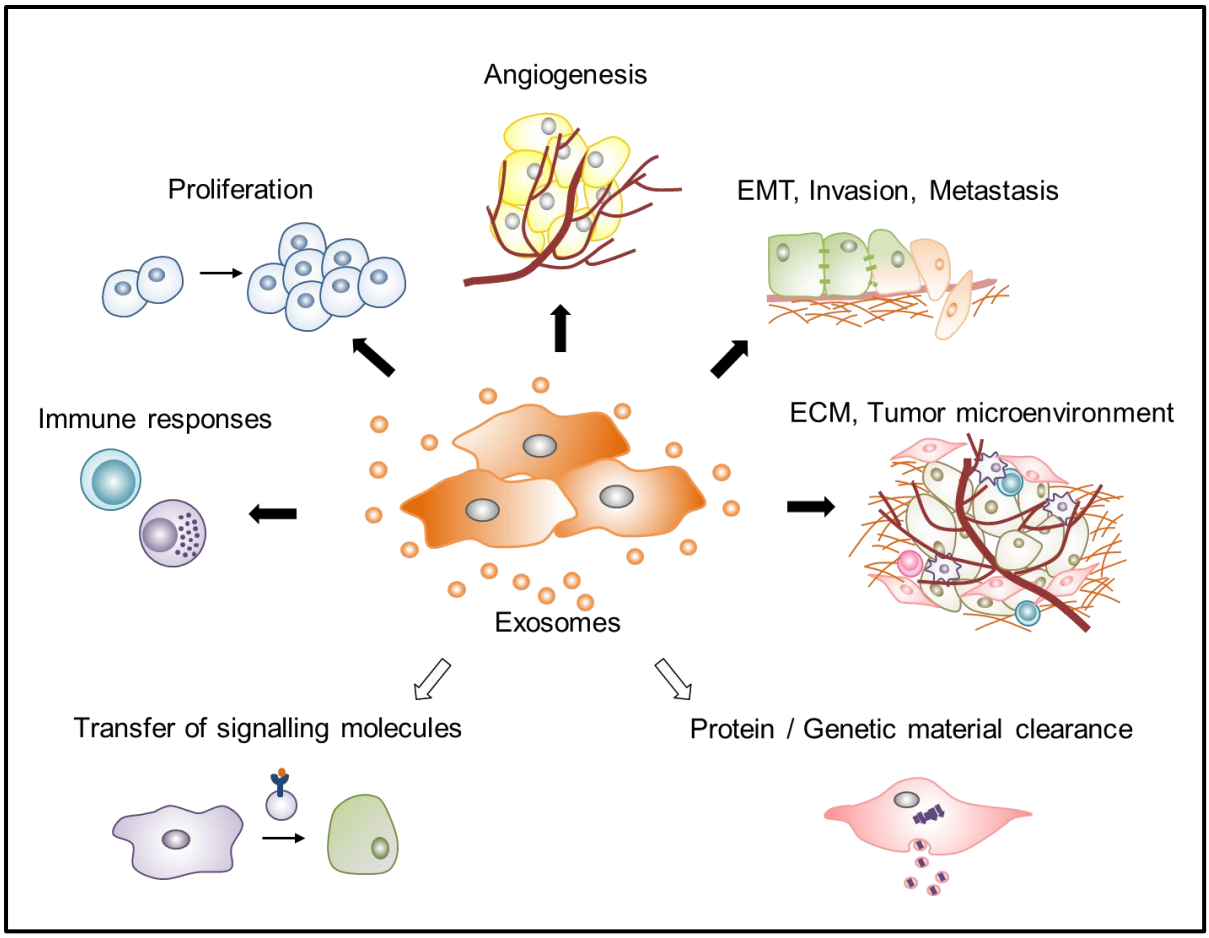
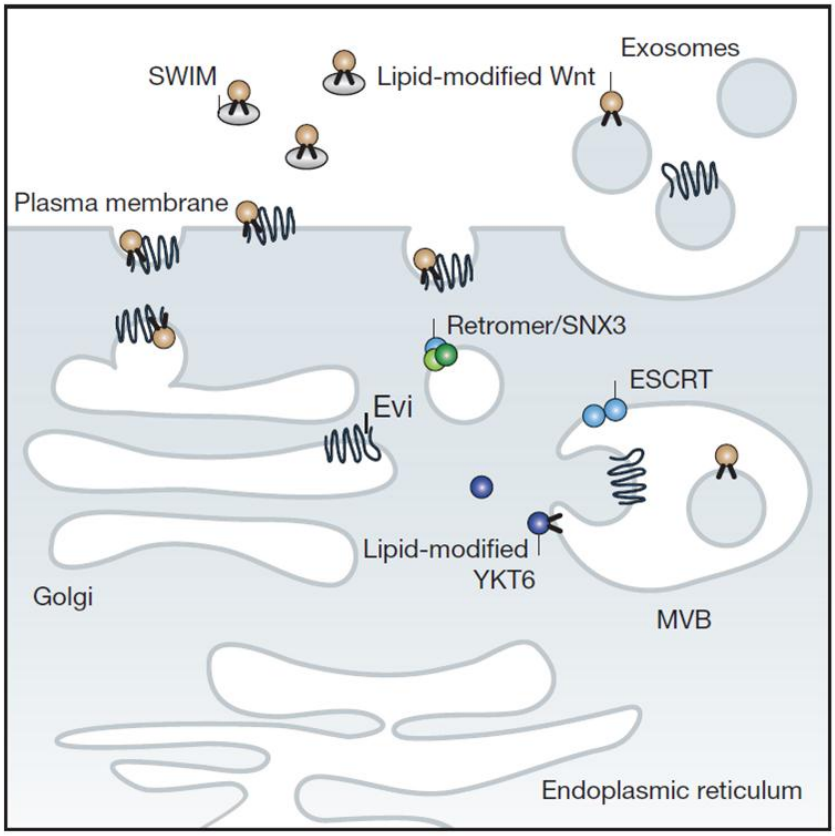


Figure 1.3. Exosomes in cancer. Exosomes affect cancer progression by regulating immune responses and facilitating tumor formation (promoting cell proliferation, invasion and metastasis, angiogenesis as well as modulating ECM and tumor microenvironment) (black arrows). Importantly, the roles of exosomes in transferring of signalling molecules and discharging cytosolic molecules (proteins and genetic materials) have been revealed (white arrows). Although some evidence has been provided, their biological effects in these two fields are far from being fully understood.

1.2.5 Signalling molecule carrier

As mentioned earlier, exosomes participate in intercellular communication via transporting signalling molecules. Notably, a study showing the secretion of active Wnt on exosomes has received most attention because Wnt signalling is the critical player in embryonic development and human diseases, particularly cancer [26]. The release of Wnt proteins on exosomes explains the paradox that the lipid-modified morphogen travel extracellularly over a distance to exert signalling activity. Active Wnt were carried on exosome surface and could activate Wnt signalling in recipient cells. The exosome release of Wnt required the ESCRT machinery, ceramide as well as R-SNARE Ykt6 (Fig. 1.4). This study suggests that the exosomal packaging of active Wnts enables Wnt signalling to spread at a distance.

Various cargoes are carried in exosomes. It was believed that the biological functions of exosomes were determined by the properties of their contents. Nonetheless, it remains to be solved whether the cargo-containing exosomes, as bioactive vesicles, act differently from their cargoes in signalling transduction. More specifically, many questions remain as to whether the ligand-containing exosomes mediate signalling activities similarly as the free soluble ligands and what is the underlying molecular nature if they show differences. Moreover, although cargo-containing exosomes have been shown to modulate tumorigenic signalling pathways (such as exosomal EGFRvIII and exosomal TGF-β) and have been proposed as potential biomarkers for tumor progression (see in 1.2.7), mechanistically, it is unclear how these tumorigenic exosomes are generated. Therefore, exploring these questions will provide sound basis for developing exosomes as a cancer biomarker. Data presented in this thesis will shed light on the functional nature of exosomal TGF-β and its potential as a tumor progression biomarker.



(Gross, J. C. et al., 2012)

Figure 1.4. Active Wnt proteins are secreted on exosomes. Active Wnts are carried on the exosome surface, which is dependent on Wnt cargo receptor Evi. The sorting of Wnt into MVBs is mediated by ESCRT machinery, ceramide and R-SNARE Ykt6.

1.2.6 Exosomal discharge of cytosolic proteins/genetic materials

For a country, its population is mainly controlled by the rates of birth and death. It has also been shown that migration and immigration contribute to the population change. Likewise, the cellular protein level is strictly controlled by protein production and degradation (or export). Keeping the balance is critical for any cell to function properly. The dysregulation of cellular protein levels has been linked to the pathogenesis of human diseases, including cancer.

(1) Classical cellular protein biosynthesis and degradation

After gene transcription, mRNA is exported from nucleus to cytoplasm, where it is translated into proteins. Ribosome is the organelle for protein biosynthesis. The amino acid-linked tRNAs bind to ribosome and move along mRNA template, resulting in the elongation of polypeptide which is terminated by stop codon (UAA, UAG, or UGA) [130]. Posttranslational modifications [131] such as folding, cutting and addition of functional groups make the newly synthesized polypeptides into mature protein products.

In eukaryotic cell, the ubiquitin-proteasome system (UPS) and lysosome are responsible for proteolytic process [132]. Three enzymatic components (E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating protein, and substrate-specific E3 ubiquitin ligase) render UPS the capacity to specifically recognize and transport target proteins for proteasomal degradation. The sorting of ubiquitinated cargoes into MVBs is the key step [133] in lysosome-mediated proteolysis. Ubiquitination of the cargo serves as sorting signal which can be recognized by ESCRT machinery. It has been demonstrated that EGFR was ubiquitinated by the ubiquitin ligase UBE4B upon associating with ESCRT-0 subunit Hrs, resulting in the subsequent endosomal sorting and lysosomal degradation [27].

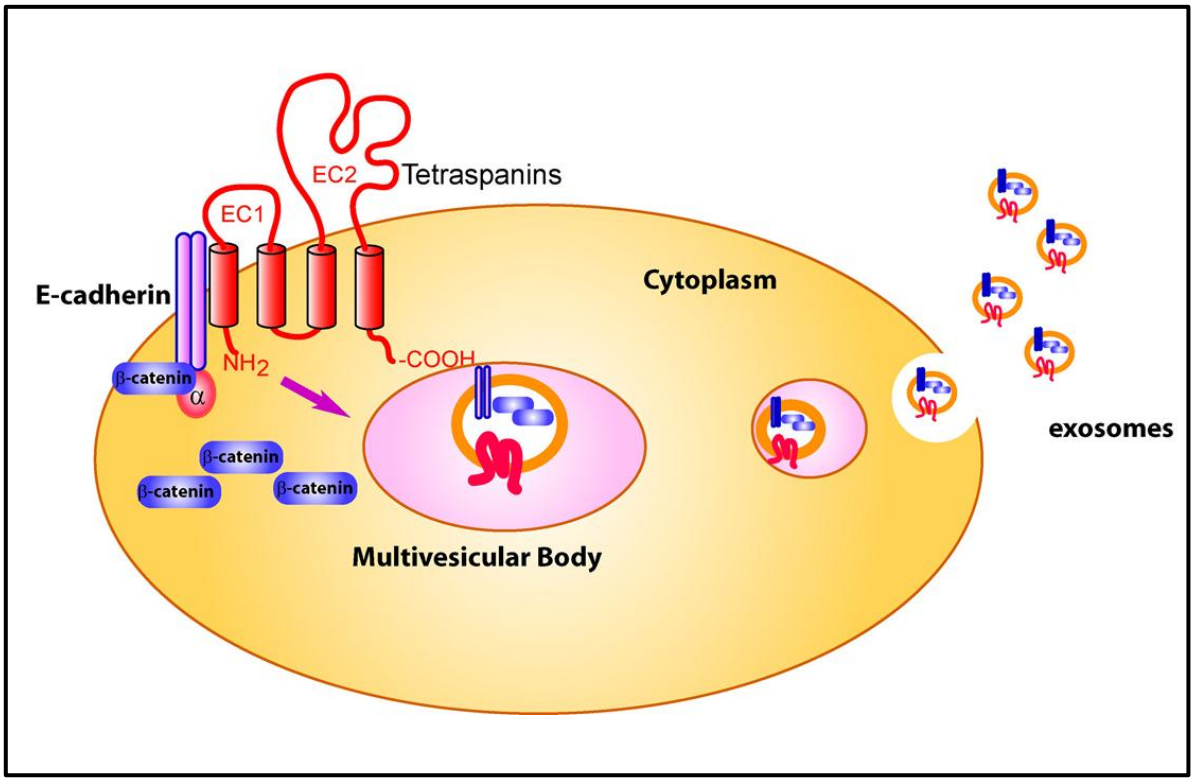
Apart from been targeted for lysosomal degradation, the ILVs of MVBs can also be released as exosomes into extracellular space when MVBs fuse with plasma membrane. As such, exosomes may provide a non-degradation mechanism for cytosolic protein discharge.

(2) Secretory exosome-mediated cytosolic protein/genetic material clearance

The protein discharge potential of exosomes was first revealed 28 years ago, when exosomes from sheep reticulocytes were found to contain the transferrin receptors (TFRs) that were disappeared during reticulocyte maturation [14]. However, little progress has been made in

this field. Until 2010, a study by Chairoungdua et al. showed that the tetraspannins CD9 and CD82 were able to attenuate the Wnt/β-catenin signalling via loading β-catenin into the secretory exosomes [18] (Fig. 1.5). In particular, the exosomal secretion of β-catenin was dependent on ceramide and the expression of E-cadherin. This study highlighted the role of exosomes in cytosolic protein clearance in addition to the proteasome- and lysosome-mediated mechanism. The tetraspannin CD63 were shown to antagonize the constitutive NF-κB activation via associating with LMP1 and enhancing the exosomal export of LMP1 from the cells [40]. In addition, the exosomal package and release of tumor suppressive miRNAs in high metastasis bladder cancer cell lines were observed to enhance the metastatic capacity of tumor cells [46].

To date, the protein and genetic material clearance potential of exosomes is far from being fully understood. This thesis provided new evidence: SPSB1, a negative regulator of TβRII, is discharged by exosome release driven by Ras expression.



(Chairoungdua, A. et al., 2010)

Figure 1.5. Exosomes mediate the discharge of β-catenin in the presence of tetraspannins CD9 and CD81. CD9 and CD81 reduce the intracellular levels of β-catenin by transporting β-catenin into secretory exosomes. The exosomal release of β-catenin is dependent on ceramide and E-cadherin. Thus, Wnt/β-catenin signalling is attenuated.

1.2.7 Therapeutic targets and cancer biomarkers

Given the critical roles of exosomes in tumor progression (interrupting immune response, promoting cell proliferation, metastasis and angiogenesis), it has attracted a great deal of interest in the strategy of targeting exosomes for cancer treatment. One of such strategies for anti-cancer therapy is to interfere with exosome biogenesis and trafficking pathways. For example, GW4869 (exosome secretion inhibitor) treatment in mice resulted in an attenuated lung multiplicities induced by Lewis lung carcinoma cells injection [134]. The shRNA-mediated knockdown of Rab27a (a key regulator in exosome release) in melanoma cells reduced exosome production and suppressed the primary tumor growth and metastasis [47]. Other studies aimed to generate therapeutic strategy by removing exosomes from the circulation [135]. However, this strategy still faces technical problems and remains theoretical. Additionally, exosomes have been recognized as specific, stable and efficient vehicles for anticancer drug delivery [136].

Numerous reports have demonstrated that exosomes expressing high level of oncogenic molecules are detectable in body fluids of cancer patients. This property of exosomes confers them the potential as an ideal biomarker for early cancer prognosis and diagnosis. In a proteomic study of urinary exosomes from normal controls and non-small cell lung cancer (NSCLC) patients, leucine-rich a-2-glycoprotein (LRG1) was found to be enriched in urinary exosomes of NSCLC patients [137]. The exosomal EGFRvIII mRNAs have also been proposed as biomarkers. The mRNAs for EGFRvIII were detectable in serum exosomes of glioblastoma patients but were absent in healthy subjects [138]. Although exosome-based diagnostic assays are currently unavailable in clinic due to the time-consuming isolation procedure [139, 140], exosomes show promising potential in cancer prognosis.

1.3 TGF-β signalling

1.3.1 TGF-β signalling pathway

(1) Ligands and receptors

TGF-β belongs to a large family of secreted dimeric cytokines, including TGF-βs, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) [141]. Three isoforms of TGF-β ligand, TGF-β1, TGF-β2 and TGF-β3 have been identified in human [142]. TGF-β is synthesized as pre-pro-protein and processed from pro-protein to small latent TGF-β complex (SLC) which is comprised of mature TGF-β and TGF-β latency associated protein (LAP). The secretion of latent TGF-β is mediated by latent TGF-β-binding protein (LTBP) [143]. SLC binds to LTBP, forming large latent TGF-β complex (LLC). After secretion, TGF-β undergoes proteolytic activation in extracellular matrix [143]. A series of latent TGF-β activators have been identified, such as proteases, thrombospondin-1, integrins, reactive oxygen species (ROS). Heat treatment and acidification also trigger TGF-β activation [144].

Active TGF-β exerts its signalling activity via transmembrane receptors, known as TGF-β type I receptor (TβRI) and TGF-β type II receptor (TβRII). In human, the TGF-β family receptor system consists of seven type I receptors (ALKs 1-7) and five type II receptors (ActR-IIa, ActR-IIB, BMPRII, AMHRII, and TβRII), pairing in different combinations. However, only TβRIIs is able to bind with TGF-β [145, 146]. TGF-β type III receptors, also known as betaglycan, bind all three soluble TGF-β isoforms and facilitate their affinity to TβRIIs [147]. In particular, Webber et al. have revealed the association between betaglycan and TGF-β on the exosome surface which is required for the exosomal transfer of active TGF-β between cancer cells [93].

Also, the endocytosis of TGF-β ligand-receptor complex has been demonstrated (Fig. 1.6). TGF-β1 undergoes endocytosis in the presence of TβRI and TβRII [95, 148, 149]. The clatherin-dependent endocytosis was found to mediate the internalization of TGF-β ligand-receptor complex and subsequently target them to early endosome, where the TGF-β/Smad signalling was enhanced. Conversely, the caveolae endocytic machinery inhibited TGF-β signalling by targeting the ligand-receptor complex to lysosome or proteasome for degradation [95]. Internalized TGF-β receptors were recycled back to plasma membrane by

Rab 11 for another round of endocytosis [150]. Because exosomes originate from endosomes, it is possible that TGF-β ligands and receptors are loaded into exosomes via endocytosis.

(2) TGF-β signalling transduction

Active TGF-β dimers bind to heterotetrameric receptor complex [151] (Fig. 1.6). The TβRI is then activated by TβRII through phosphorylation on its 'GS sequence'. The phosphorylated TβRI propagates the signalling through recruiting downstream signalling molecules [152].

It is well documented that Smad proteins are recruited and phosphorylated in response to canonical TGF-β signalling. Smads are small intracellular proteins containing Mad homology domains MH-1 and MH-2 at their N-terminals and C-terminals, respectively. The MH-1 domain and MH2 domain is linked by a sequence which can be phosphorylated by kinases and can engage ubiquitin ligases [146]. The Smad proteins are divided into three subgroups according to their function. The first group is defined as the receptor-regulated Smads (R-Smads) [153], including Smad1, 2, 3, 5 and 8, while Smad6 and 7 are grouped to the inhibitory Smads (I-Smads) [154]. The common mediator Smad4 (Co-Smad) is incorporated into transcriptional complex upon R-Smads activation.

The binding of TGF-β to the TβRI and TβRII receptor complex triggers the activation of TβRI kinase activity, thereby phosphorylating R-Smads (Smad2 and Smad3). Once phosphorylated, R-Smads form heterotrimeric complexes with Smad4 and subsequently translocate into the nucleus where they activate or repress the transcription of target genes by associating with transcription factors [155-157] (Fig. 1.6).

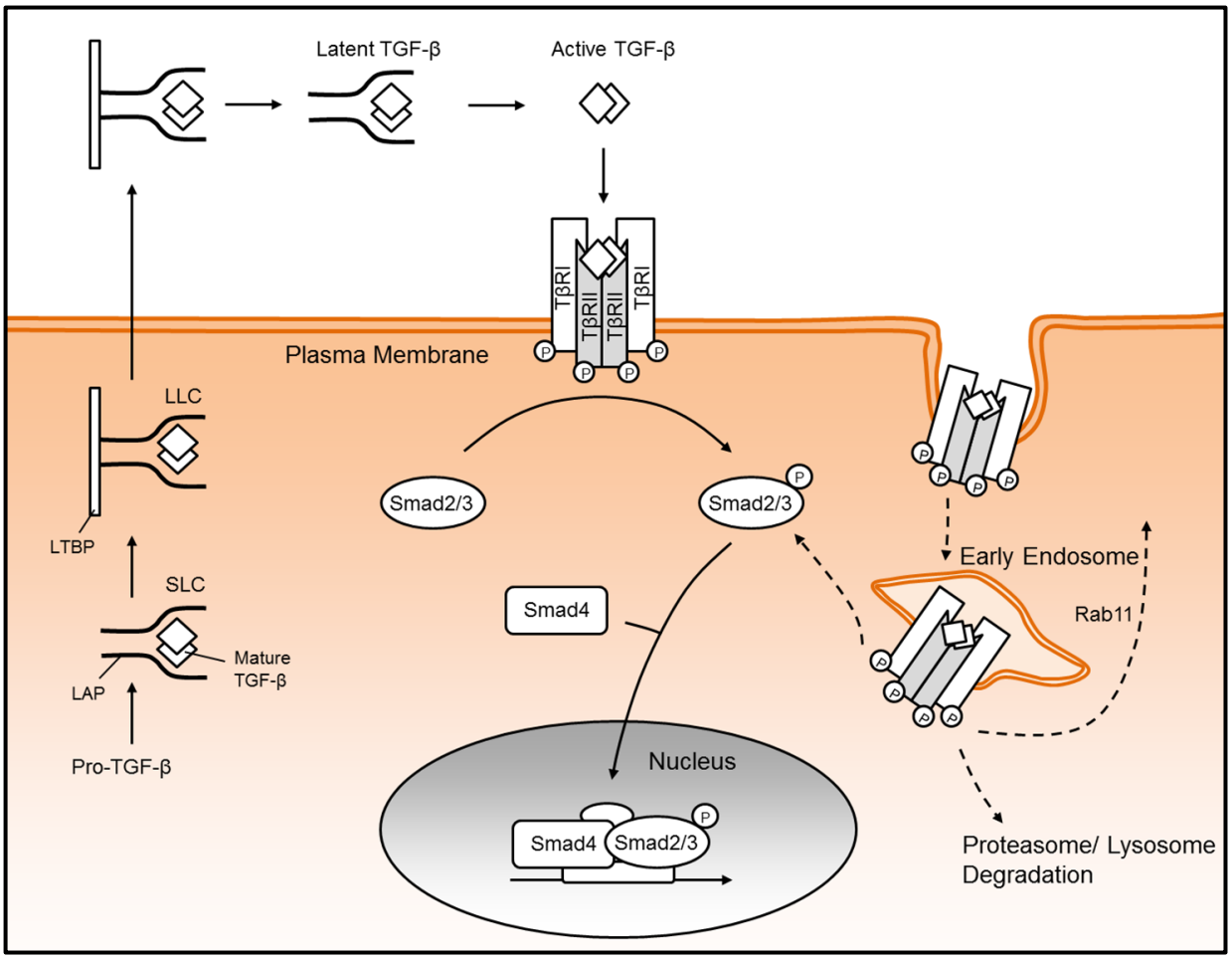


Figure 1.6. TGF-β signalling. Latent TGF-β is activated in extracellular matrix. Active TGF-β dimers bind to heterotetrameric receptor complex. The activated TβRI and TβRII receptor complex phosphorylates R-Smads (Smad2 and Smad3). Once phosphorylated, R-Smads form heterotrimeric complexes with Smad4 and translocate into the nucleus where they activate or repress the transcription of target genes by binding to transcription factors. Also, active TGF-β ligand-receptor complexes are found to undergo clatherin- or caveolae-dependent endocytosis (dashed lines). They are transported to early endosome to phosphorylate R-Smads or to lysosome and proteasome for degradation. Internalized TGF-β receptors can also be recycled back to plasma membrane by Rab 11 for another round of endocytosis. LAP, TGF-β latency associated protein. SLC, small latent TGF-β complex. LTBP, latent TGF-β-binding protein. LLC, large latent TGF-β complex. TβRI, transforming growth factor-β type I receptor. TβRII, transforming growth factor-β type II receptor.

1.3.2 TGF-β signalling is tightly regulated

As a key player in various biological processes, TGF-β signalling is tightly regulated by multiple molecules at each step of the signalling cascade (Fig. 1.7). Dysregulation of TGF-β signalling has been implicated in the pathogenesis of human diseases.

(1) Controlled TGF-β secretion and activation

As mentioned above, TGF-β is processed from pro-protein to SLC before the formation of LLC. Emilin-1 and E-selectin ligand-1 (ESL-1) have been shown to suppress this process [158]. Loss of Emilin-1 has been linked to increased blood pressure in a mouse model [159]. ESL-1 suppressed the maturation of TGF-β precursors by retaining them in Golgi apparatus [160]. ESL-1 deficiency resulted in an increased TGF-β signalling and a subsequent delayed differentiation of chondrocytes. Upon LLC secretion, Fibrillin-1 protected TGF-β from proteolytic cleavage and thus reduced TGF-β signalling [161]. Several glycoproteins are involved in the regulation of active TGF-β. For example, Decorin bound directly to TGF-β ligands and neutralized their activity [162]. The α_2 –macroglobulin had a high affinity for active TGF-β, and could inhibit TGF-β signalling in vivo [163].

Of note, active TGF-β has been discovered in exosomes, a crucial mediator in intercellular communication. Exosomal TGF-β is shown to activate the Smad-dependent signalling in recipient cells and drive fibroblast to myofibroblast differentiation [93]. However, how exosomes are involved in the regulation of TGF-β signalling is not clear. As carriers of signalling molecules, exosomes enable the lipid-modified Wnt ligands to travel extracellularly over a distance and exert signalling activity in recipient cells [26]. On the other hand, exosomes are involved in the inhibition of Wnt signalling by mediating the discharge of cytosolic β-catenin [18]. Is there a parallel exosomal regulation of signalling between Wnt and TGF-β?

(2) Regulation of TGF-β receptor activation

Active TGF-β dimers bind to heterotetrameric receptor complex, resulting in phosphorylation and activation of the receptor complex. TβRI was found to be dephosphorylated by protein phosphatases, such as protein phosphatase 1 (PP1) [164]. The formation of PP1 holoenzyme was mediated by Smad7, which recruited PP1 catalytic subunit PP1c to the regulatory subunit GADD34. Thus, Smad7-induced PP1 contributed to TGF-resistance by dephosphorylating TβRI [165]. The protein phosphatase 2A (PP2A) regulatory subunit Bδ, was identified to

restrict TβRI activity whereas the subunit Bα stabilized TβRI and enhanced TGF-β signalling [166]. TβRII was reported to be dephosphorylated by the T cell protein tyrosine phosphatase (TCPTP) in an integrin α1β1-dependent manner. Loss of integrin α1β1 in mice led to severe renal fibrosis due to up-regulation of TGF-β–induced fibrotic signalling [167].

The activity of TGF-β receptors is also controlled by ubiquitination-mediated degradation. In particular, Smad7 is required for the ubiquitination of TβRI. In response to TGF-β, Smad7 recruited Smad ubiquitin regulatory factors (Smurf1 and Smurf2), two E3 ubiquitin ligases, to TβRI and targeted it to proteasome for degradation [168, 169]. Also, Smad7 mediated the association between TβRI and E3 ligases such as neuronal precursor cell-expressed developmentally downregulated 4-2 (Nedd4-2) [170] and WW domain containing E3 ubiquitin protein ligase 1 (WWP1) [171]. Multiple molecules are involved in Smad7-induced TβRI ubiquitination. Salt-inducible kinase (SIK), a target gene of TGF-β signalling, promoted the degradation of TβRI by enhancing Smad7-TβRI complex formation. Thus, SIK controlled TGF-β signalling through a negative feedback loop [172, 173]. Atrophin 1-interacting protein 4 (AIP4/Itch), was shown to bind to Smad7 and enhanced the recruitment of Smad7 to TβRI [174]. Conversely, the ubiquitination of TβRI is balanced by deubiquitinating enzymes, such as ubiquitin C-terminal hydrolase 37 (UCH37). UCH37 interacted with Smad7 and stabilized TβRI, thus increasing TGF-β signalling [175]. Ubiquitin-specific proteases, USP4 and USP15, were identified to activate TGF-β signalling through deubiquitinating and stabilizing TβRI [176, 177]. Also, Hsp90 bound to TβRI and TβRII and protected them from Smurf2-mediated proteasome degradation [178].

Apart from proteasome-dependent degradation, TβRI can be targeted to lysosome pathway. It has been shown that Dapper 2 interacted with TβRI and transported TβRI to lysosome for degradation. Whether ubiquitination is required in this process is not clear [164] [179]. In addition, TβRIs have been found to undergo sumoylation [180]. Sumoylation of TβRI by Ubc 9 resulted in increased TGF-β signalling by enhancing the recruitment and phosphorylation of R-Smads.

Although the tight control of TβRIs is extensively studied, little is known about the regulators of TβRIIs. Sheng Liu, a PhD student in our laboratory first identified SPSB1, a SPRY domain-containing SOCS box protein, as a novel negative regulator of TGF-β signalling [181]. SPSB1 directly interacted with TβRII via its SPRY domain, facilitating the

ubiquitination and degradation of TβRII. Given that SPSB1 is a direct transcriptional target of TGF-β signalling, it limits TGF-β signalling in a negative feedback loop.

(3) Regulated Smad activity

TGF-β signalling is transduced from the cell surface to nucleus by Smad proteins. Upon TβRI activation, R-Smads (Smad2 and Smad3) are recruited and phosphorylated. Smad archor for receptor activation protein (SARA) acted as an adaptor protein in R-Smad activation by facilitating the binding of Smad2/3 to activated TβRI [182]. The interaction between R-Smads and scaffold proteins (such as SARA and cPML) could be enhanced by PML competitor for TGIF association (PCTA) [183]. Conversely, BAMBI was shown to interfere with the association between R-Smads and TβRI [184]. Similarly, TMEPAI, a transcriptional target of TGF-β, protected R-Smads from binding to TβRI, thus blocking TβRI-mediated R-Smad phosphorylation [185]. R-Smads are phosphorylated by activated TβRI at the C-terminal SSXS motif [163]. Multiple phosphatases (such as protein phosphatase 1A, PPM1A) have been implicated in the termination of TGF-β signalling by dephosphorylating SSXS motif of Smad2/3 [186]. PTEN, the tumor suppressor that inhibits PI3K/Akt signalling, was reported to maintain the stability of PPMA1 [187, 188]. The phosphatase Myotubularin-related protein 4 (MTMR4) has been linked to dephosphorylation of R-Smads in early endosomes. Also, MTMR4 sequestered active Smad3 in early endosomes, preventing their nuclear translocation. Consequently, TGF-β signalling was attenuated [189]. In addition, ubiquitination-mediated degradation is involved in the termination of TGF-β signalling. For example, Nedd4L triggered the poly-ubiquitination and degradation of Smad2/3 through recognizing TGF-β-induced phosphorylation in the linker region [190]. Other ubiquitin ligases such as Smurf1, Smurf2, Nedd4–2, WWP1, ROC1-SCF, and C-terminus of Hsc70 interacting protein (CHIP) are also involved in the degradation of R-Smads [191].

Once phosphorylated, R-Smads form heterotrimeric complexes with Smad4 and subsequently translocate into the nucleus where they activate or repress the transcription of target genes by associating with transcription factors [155-157]. The oncogenic proteins Ski and SnoN have been suggested to compete with R-Smads for binding to Smad4 and also to interfere with the association between R-Smads and transcriptional co-activator p300/CBP, therefore suppressing the TGF-β signalling [192, 193]. Smad4 could be poly-ubiquitinated by Ectodermin and be targeted to proteasome for degradation. Ectodermin also mediated the mono-ubiquitination of Smad4, thus abrogating its interaction with active R-Smads [188].

Apart from inhibiting R-Smads phosphorylation [194], Smad7 is found to compete with Smad4/R-Smads complex for binding to target gene or target Smad4 for degradation by recruiting Smurf1, Smurf2, Nedd4–2, WWP1 [195], thus inhibiting TGF-β signalling. A study by Bourgeois et al. has shown that the nuclear membrane protein MAN1 competed with transcription factors for interacting with Smad2/3, resulting in the dephosphorylation of R-Smads by phosphatase PPM1A [196]. The tumor suppressor LKB1 prevented Smad4 from binding to target gene via mediating the phosphorylation on its DNA-binding domain [197].

As an important antagonist of TGF-β signalling, the level of Smad7 is tightly regulated by multiple molecules. Smad7 is directly induced by TGF-β signalling, thus controlling TGF-β signalling in a negative feedback loop. Cytokines including IFN-γ, IL-7, and TNF-α can also stimulate the expression of Smad7 [188]. Smad7 is negative regulated by miRNAs. In particular, *miR-21*-induced reduction of Smad7 has been linked to lung fibrosis [198]. Ubiquitination-mediated degradation also participates in Smad7 regulation. For example, Arkadia was shown to mediate the ubiquitination and degradation of Smad7, therefore enhancing TGF-β signalling. RNF12, an E3 ligase, was found to control TGF-β signalling by targeting Smad7 for degradation [199]. Furthermore, Smad7 level is controlled by acetylation. The acetylation of Smad7 maintains its stabilization by protecting it from ubiquitination-mediated degradation [188].

(4) Dysregulation of TGF-β signalling in disease

TGF-β signalling, a key player in a broad range of biological processes is tightly regulated by multiple molecules at each step of the signalling cascade. Dysregulation of TGF-β signalling has been implicated in the pathogenesis of disease. Loss of fibrillin-1, a negative regulator in TGF-β ligand activation, has been linked to Marfan syndrome (MFS) [200]. BAMBI-deficient mouse model showed enhanced TGF-β signalling endothelial dysfunction [201]. The mutation in Ski (Smad interacting protein) contributed to Shprintzen-Goldberg syndrome (SGS) by inducing excessive TGF-β signalling activation [202]. Apart from defects or mutations in regulators, diseases caused by loss of TGF-β signalling are also observed. For example, highly expressed SnoN antagonized TGF-β-induced cell cycle arrest in Polyoma middle T antigen (PyVmT) tumor, thus promoting tumor progression. The *WWP1* gene was reported to be amplified in prostate and breast cancers while high copy numbers of *NEDD4-2* were observed in prostate and bladder cancers. Also, colorectal and breast cancers express high levels of Ectodermin, the ubiquitin ligase that target Smad4 [173]. Given the critical

roles of TGF-β signalling regulators in disease pathogenesis, it will be important to expand our knowledge about regulators of TGF-β signalling which will aid the development of novel therapeutic strategies.

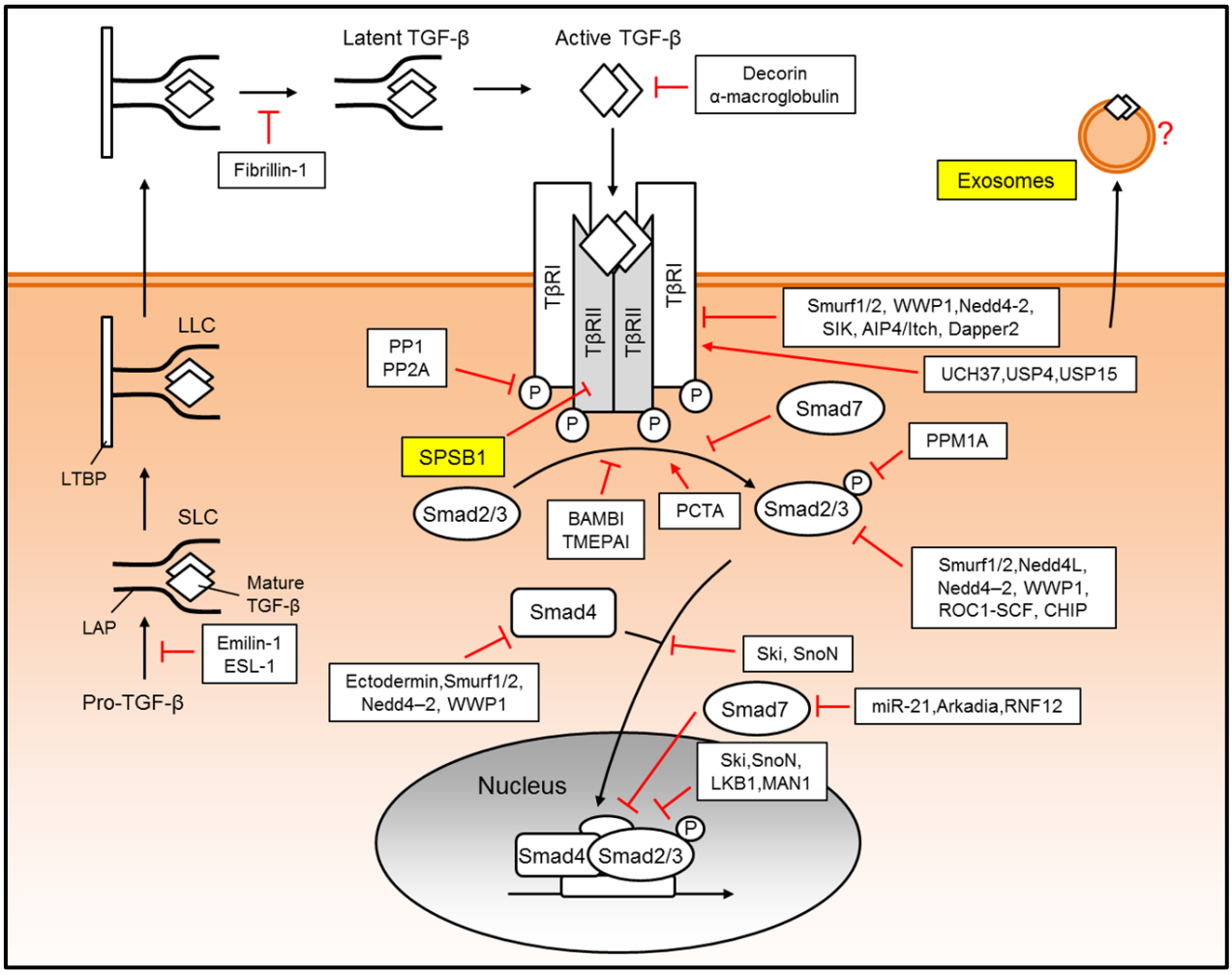
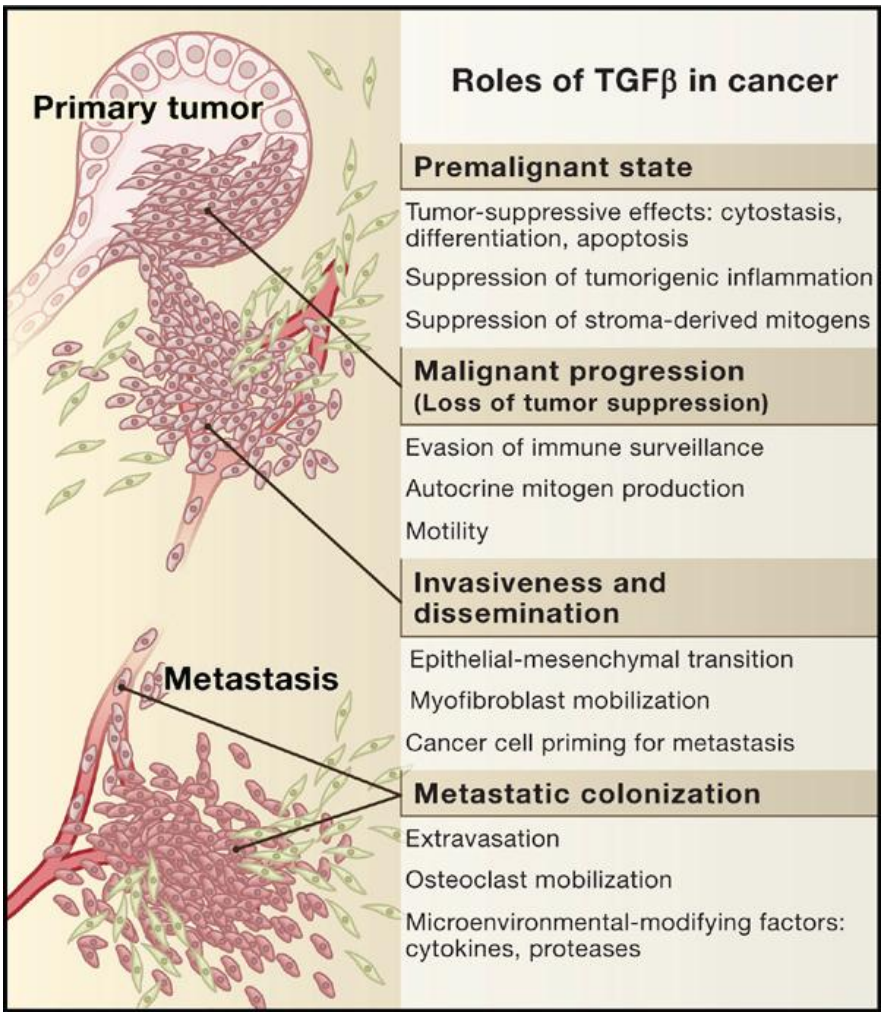


Figure 1.7. TGF-β signalling is tightly regulated. TGF-β signalling is tightly regulated by multiple molecules at each step of the signalling cascade. In particular, SPSB1 is the only negative regulator that target TβRII to proteasome for degradation. Although active TGF-β has been discovered in exosomes, how exosomes may participate in the regulation of TGF-β signalling is yet to be investigated. ESL-1, E-selectin ligand-1. PP1, protein phosphatase 1. PP2A, protein phosphatase 2A. SPSB1, SPRY domain and SOCS Box containing protein 1. PCTA, PML competitor for TGIF association. Smurf1/2, Smad ubiquitin regulatory factor 1 and 2. WWP1, WW domain containing E3 ubiquitin protein ligase 1. Nedd4-2, neuronal precursor cell-expressed developmentally downregulated 4-2. SIK, salt-inducible kinase. AIP4/Itch, Atrophin 1-interacting protein 4. PPM1A, protein phosphatase 1A. CHIP, C-terminus of Hsc70 interacting protein. UCH37, ubiquitin C-terminal hydrolase 37. USP4 and USP15, ubiquitin-specific proteases 4 and 15. LAP, TGF-β latency associated protein. SLC, small latent TGF-β complex. LTBP, latent TGF-β-binding protein. LLC, large latent TGF-β complex. TβRI, transforming growth factor-β type I receptor. TβRII, transforming growth factor-β type II receptor.

1.4 TGF-β signalling in cancer

It is well documented that TGF-β plays a dual role in cancer. In normal or premalignant cells, TGF-β exerts the tumor-suppressive effects via regulating proliferation, apoptosis, cytostasis, or through manipulating tumor stroma. In a large subset of malignant tumors (such as colorectal, ovarian, gastric and pancreatic carcinomas), the inactivation or deletion of core TGF-β signalling components are observed. However, highly aggressive tumors (such as breast cancer, melanoma and prostate cancer) are able to circumvent the tumor-suppressive effects of TGF-β while still preserve the core pathway components. In this case, tumor cells use TGF-β signalling to their advantage, promoting invasion, metastasis and also immune evasion [141, 146] (Fig. 1.8).



(Massague, J. 2008)

Figure 1.8. TGF-β signalling plays a dual role in cancer. In normal or premalignant stage, TGF-β signalling exerts the tumor-suppressive functions. However, highly aggressive tumors circumvent the tumor-suppressive effects of TGF-β signalling while use TGF-β signalling to their advantage, promoting invasion, metastasis and immune evasion.

1.4.1 TGF-β signalling as tumor suppressor

TGF-β is a potent inhibitor of malignant progression. It has been revealed that expression of TβRII in human breast cancer MCF-7 cells resulted in reduced tumorigenicity [203], while mutations in TGF-β receptors and Smad proteins were frequently observed in colorectal, breast, head and neck, ovarian and pancreatic cancers. In particular, mutational inactivation of TβRII was commonly found in human colorectal cancer with microsatellite instability, which could bypass the growth-inhibitory effects of TGF-β [204]. TGF-β-insensitive human ovarian tumors harboured TβRIs, TβRIIs mutations with the loss of Smad4 [205]. More recently, it has been demonstrated in Smad4-deficient mouse model that Smad4 correlated with the formation of human head and neck squamous cell carcinoma (HNSCC) and increased genomic instability [206]. Also, using colon cancer mouse models, it was found that the deficiency of Smad2 and Smad3 contributed to accelerated tumorigenesis [207].

TGF-β signalling has been shown to control the cytostatic and apoptotic responses. TGF-β signalling modulates cell proliferation by inducing cyclin-dependent kinases (CDK) inhibitors and repressing the oncogenes which enhance cell growth. Upon activation of TGF-β signalling, the downstream Smad proteins (Smad3 and Smad4) formed complexes with FoxO transcription factors, bound to the promoters of p15INK4b and p21CIP1 (two CDK inhibitors) and activated their transcription [208]. 4E-BP1, a translation inhibitory protein, was identified as a target of Smad4 upon TGF- β stimulation [209]. On the other hand, oncogene *c-Myc* was found to be suppressed by the TGF-β -induced protein complex containing Smad3, Smad4 and C/EBP β [210]. Moreover, TGF-β signalling can trigger apoptosis by promoting pro-apoptotic responses while interrupting pro-survival activities. For example, FoxO3 and pro-apoptotic protein Bim have been shown to take part in TGF-β-induced apoptosis in hepatocarcinoma cells [211]. FoxO3 was activated by TGF-β and formed a complex with Smad2 and Smad3, resulting in the up-regulation of Bim and the subsequent cell apoptosis.

TGF-β has also been implicated in the control of cell differentiation. TGF-β signalling drove the precursor cells to a less proliferative state, thus enhancing cell differentiation as well as blocking tumor progression [212]. The nuclear IκB kinase α (IKKα) was required for TGF-β-mediated keratinocyte differentiation [213]. Also, TGF-β was able to induce the senescence of stem cell, and attenuate their self-renewing capability which was responsible for tumor development [214].

Apart from the direct inhibitory effects on tumor cells, TGF-β can restrict tumor progression through modulating the tumor stroma [215]. The oral squamous cell carcinoma (OSCC) and oral carcinoma-associated fibroblasts (CAFs) showed lower levels of TGF-β receptors compared with oral premalignant lesions (oral leukoplakia, OLK) [216]. The loss of TGF-β receptors was also observed in the stroma of prostate tumor, which could drive the malignant progression of nontumorigenic cells [217]. Moreover, TGF-β plays an important role in suppressing destructive immune and inflammatory reactions. TGF-β was identified to enhance the immune tolerance in the intestinal mucosa and thus decrease the risk of colon cancer [218].

1.4.2 TGF-β signalling as tumor promoter

Aggressive cancer cells that bypass the tumor-suppressive effects of TGF-β can exploit TGF-β signalling to their advantage and promote the tumor progression. It has been shown in a breast cancer mouse model that blocking TGF-β isoforms by neutralizing antibodies suppressed tumor spread [219]. Additionally, targeting the kinase activity of TGF-β receptors inhibited the invasion and metastasis of hepatocellular cancer cells [220]. An inhibition of EMT was observed when TGF-β signalling was blocked by Smad-binding peptides [221].

TGF-β has been well characterized as a predominant player in EMT. During EMT, cells lose the epithelial properties, while acquire increased motility and invasiveness. Mechanistically, EMT is elicited by a set of transcription factors, including zinc-finger proteins Snail and Slug, the zinc-finger/homeodomain proteins ZEB-1 and -2 and the bHLH factor Twist [146]. Studies have revealed the induction of these transcription factors in response to TGF-β, such as Snail. The expression of mesenchymal genes was also observed upon TGF-β stimulation. Additionally, TGF-β drives EMT by cooperating with other signalling pathways, such as RHO-like GTPases, and PI3K pathways. TGF-β was shown to activate PI3K/AKT signalling while inactivate GSK3β, resulting in the stabilization of Snail1 and a decreased expression of the epithelial markers. EMT induced by TGF-β and MAPK/ERK pathway has been demonstrated, which was further enhanced by Ras signalling [212, 222].

Studies have also shown the dependency of cancer cell invasion, migration and metastasis on TGF-β signalling. For example, TGF-β contributed to the invasion of breast cancer cell by inducing the complexes containing Smad proteins and transcription factor AP-1components, which enabled the expression of invasion-associated genes [223]. Also, augmented α-SMA expression and cell migration were observed in response to TGF-β stimulation [224]. High

TGF-β levels have been linked to poor outcome in colorectal cancer (CRC). TGF-β was found to control tumor metastasis via stimulating the secretion of IL-6 in the tumor stroma of CRC [225]. Also, altered TGF-β in breast cancer cells led to increased levels of miR-181a , which were capable of driving pro-metastatic responses and were considered as biomarkers and therapeutic targets for breast cancer treatment [226]. Moreover, TGF-β was identified as a key factor in leucine-rich alpha-2-glycoprotein 1 (Lrg1)-induced angiogenesis [227].

Immune evasion is another downstream event of TGF-β signalling that provides beneficial effects on tumor progression. TGF-β was shown to block IL-15-mediated activation of NK cells in tumor microenvironment. Inhibition of TGF-β signalling could restore the function of NK cells [228]. TGF-β could also antagonize antitumor immunity by affecting T cell development in brain tumor [229].

1.4.3 Crosstalk between TGF-β signalling and Ras signalling

As mentioned earlier, TGF-β can signal not only via linear signalling cascade but also through the crosstalk with the other pathways, including PI3K/AKT, WNT, Hedgehog (HH), Notch, interferon (IFN) and TNF pathways [230]. For example, PI3K/AKT was shown to interfere with TGF-β signalling by restricting Smad3 activation. Also, an increase in the activity of PI3K/AKT signalling was observed after TGF-β treatment [231]. Wnt signalling synergized with TGF-β signalling to induce the expression of connective tissue growth factor (CTGF), a secreted protein which enhanced the binding of TGF-β ligands to receptors [232]. In addition, TGF-β was demonstrated to block IL-15-mediated activation of NK cells in tumor microenvironment [228], thus triggering immune evasion. Here we pay particular attention to the crosstalk between TGF-β signalling and Ras signalling, the two most important pathways in cancer development.

(1) Ras signalling

Ras proteins belong to the superfamily of small GTPase. As molecular switches, Ras proteins cycle between the GTP-bound active form and the GDP-bound inactive form under the control of guanine nucleotide exchange factors (GEFs). Conversely, Ras is inactivated by GTPase-activating proteins (GAPs). *RAS* genes have been well characterized as the most important oncogenes. The mutations in *RAS* genes lock Ras in the GTP-bound form which is insensitive to GAP, conferring them oncogenic property.

The oncogenic mutations in three Ras isoforms, H-Ras, K-Ras and N-Ras, are ubiquitously detected in human cancers [233]. K-Ras mutations are found in 90% pancreatic carcinomas, while leukemias display high frequency of N-Ras mutation [234, 235]. All these three Ras isoforms are present at plasma membrane. K-Ras transiently associate with endosomes, while H-Ras and N-Ras are found at Golgi and endosomes. The different subcellular distributions of Ras are related to their signalling outputs. For example, the Rabex-5-mediated ubiquitination targeted H-Ras to endosome, where the Ras signalling was enhanced [236, 237]. Ras proteins was able to recruit PI3K to endosome and resulted in PI3K activation in this compartment [36]. Of note, apart from their intracellular distribution, the exosome secretion of K-Ras, N-Ras and H-Ras has been demonstrated [74].

Studies have revealed the mechanism by which Ras signalling is activated. Upon extracellular stimuli (growth factors such as EGF), the membrane protein tyrosine kinase receptors (PTKRs) undergo autophosphorylation and recruit Src homology 2 (SH2)-domain-containing proteins. The SH2 domain-containing adaptor protein Grb2 form complexes with the Ras exchange factor SOS, leading to the activation of Ras and the subsequent initiation of cascade Raf-MEK-ERK pathway [238]. Thus, Ras proteins appear as pivotal signalling transducers that modulate multiple cellular contexts, including cell proliferation, cell invasion and cell survival.

(2) Crosstalk between TGF-β signalling and Ras signalling

It has been suggested that the resistance of tumor cells to tumor-suppressive function of TGF-β signalling may be conferred by oncogenic Ras. For example, Ras-induced activation of MAPK/ERK signalling was shown to suppress the expression of TGF-β receptors in lung cancer [239]. Ras signalling has also been found to antagonize the tumor-suppressive function of TGF-β signalling via mediating the cytoplasmic retention of Smad proteins [240]. Moreover, Ras signalling was essentially involved in the ability of hepatocarcinoma cells to bypass TGF-β-induced cell apoptosis. The Ras-activated MAPK/ ERK pathway impaired the up-regulation of the NADPH Oxidase NOX4 which was required for TGF-β-mediated cell death [241].

On the other hand, Ras signalling is demonstrated to synergize with TGF-β signalling by enhancing tumorigenic effects of TGF-β. Sustained activation of Ras signalling results in EMT, which is maintained by Ras-mediated autocrine loop of TGF-β [242, 243]. Mechanistically, active Ras cooperated with TGF-β in inducing Snail expression, allowing

epithelial cells to undergo EMT [244]. Also, the nuclear localization of ERK2 as well as the activation of c-Myc was observed in human prostate cancer cells harbouring oncogenic Ras, which was responsible for TGF-β-induced EMT and cell invasion [245]. Additionally, TGF-β was shown to facilitate ^{V12}HaRas-mediated transformation and anchorage-independent growth by activating RhoA signalling [246].

(3) SPSB1 bridges TGF-β signalling and Ras signalling

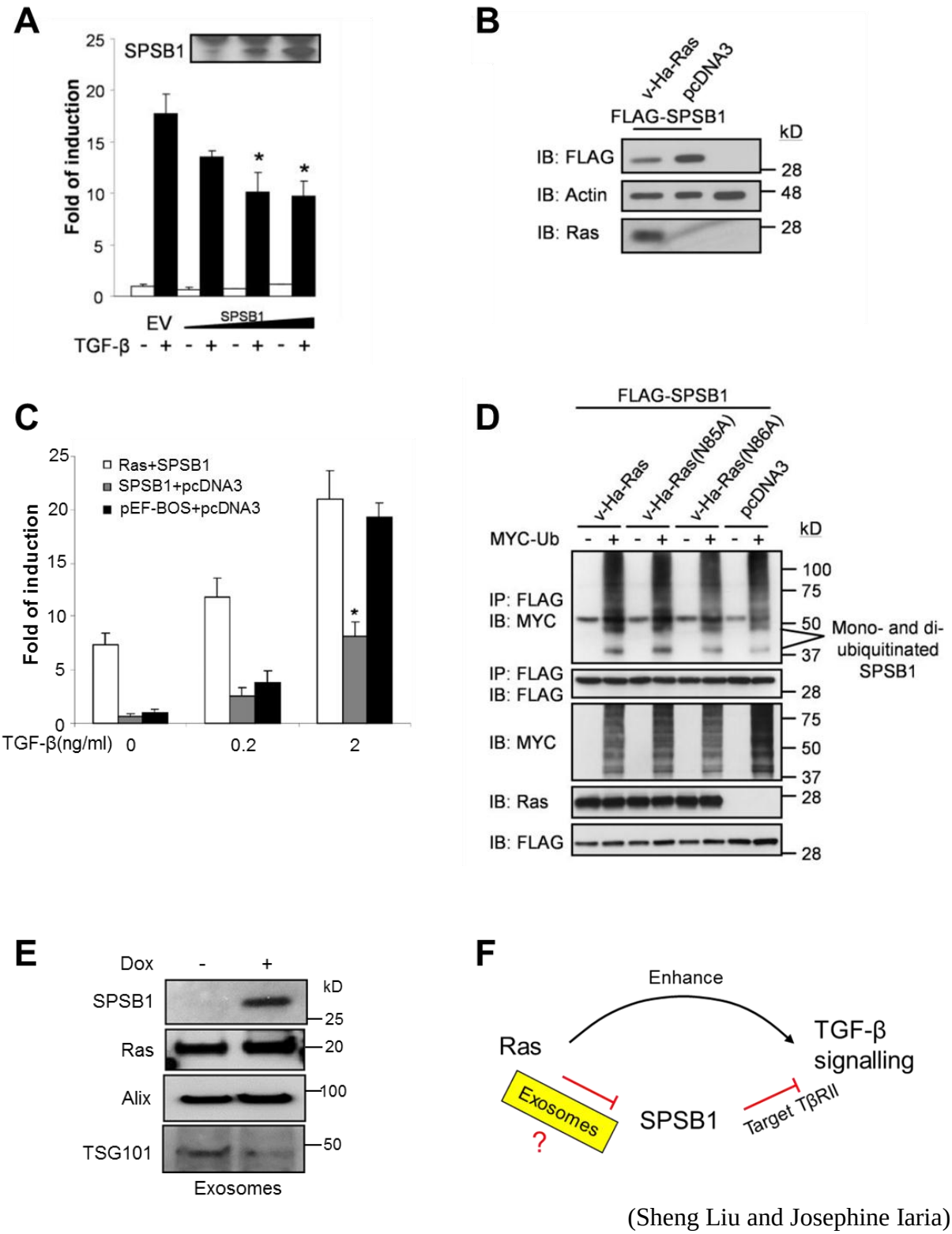
Although TGF-β signalling and Ras signalling have been found to act in synergy to promote tumor progression, the molecular mechanisms by which Ras signalling may enhance TGF-β signalling are not well understood. Sheng Liu, a PhD student in our laboratory first identified that SPRY domain and SOCS Box containing protein 1 (SPSB1) bridges between Ras signalling and TGF-β signalling, contributing to Ras-induced increase in TGF-β signalling activity.

SPSB1 belongs to the SPRY domain and SOCS box-containing protein family, which consists of four members, SPSB1, SPSB2, SPSB3 and SPSB4. Structurally, they are comprised of an N-terminal SPRY domain and a C-terminal SOCS box and were first identified in 1998 as subfamily members of the suppressor of cytokine signalling (SOCS) protein family [247, 248]. The SPRY domain has been recognized as a protein interaction module which regulates innate and adaptive immunity [249]. The SOCS box binds to elongin B and elongin C, cullin-5 and RING-box-2 (RBX2), resulting in the recruitment of E2 ubiquitin transferase. Several SOCS box containing proteins have been found to act as E3 ubiquitin ligases. For example, SOCS1 targeted JAKs to MTOC-dependent proteasome degradation [250].

In 2005, Valcourt et al. identified *SPSB1* as a TGF-β target gene by transcriptomic profiling [251]. Sheng's data suggested that SPSB1 negatively regulates TGF-β signalling (Fig. 1.9 A) [181]. Overexpression of SPSB1 in a range of cell lines (such as NIH3T3, HEK 293T, MDCK and U87MG) led to 50% decrease in TGF-β signalling activity. Mechanistically, SPSB1 interacted with TβRII (rather than TβRI) via its SPRY domain and mediated the ubiquitination and degradation of TβRII through its SOCS box. SPSB1 knockdown resulted in enhanced TGF-β signalling as well as tumor cell invasion and migration. Thus, SPSB1 appears to be a negative regulator of TGF-β signalling by targeting TβRII to proteasome for degradation [181].

In addition, Sheng's data showed that SPSB1 directly interacted with Ras via its SPRY domain. More importantly, Ras expression resulted in a reduction in the intracellular levels of SPSB1 (Fig. 1.9 B), which in turn stabilized TβRII and triggered elevated TGF-β signalling activity (Fig. 1.9 C). Therefore, Ras enhances TGF-β signalling via targeting SPSB1, a TβRII negative regulator, for degradation (Liu et al., manuscript in reviewing).

Interestingly, although intracellular SPSB1 level decreased after Ras expression, the poly-ubiquitination of SPSB1 remained unchanged. However, an increase in the mono (1×)-, double (2×)- and triple (3×)-ubiquitination of SPSB1 was observed in the presence of Ras (Fig. 1.9 D). Apart from the canonical poly-ubiquitination-mediated proteasomal degradation, mono-ubiquitination has been reported to act as a signal for MVBs sorting and the subsequent exosomal secretion [252-255]. Also, the data by Josephine Iaria in our laboratory showed the doxycycline-induced expression of SPSB1 in the exosomes from 21D1 (Ras-transformed MDCK cells) cells (Fig. 1.9 E). Given that exosomes contribute to reduced Wnt signalling via mediating the discharge of cytosolic β-catenin [18], it is possible that exosomes are involved in the Ras-induced up-regulation of TGF-β signalling by exporting SPSB1 from the cell.



(Sheng Liu and Josephine Iaria)

Figure 1.9. SPSB1 bridges TGF-β signalling and Ras signalling. (A) SPSB1 is a negative regulator of TGF-β signalling. HEK 293T cells were co-transfected with TGF-β reporter pCAGA12-luc and SPSB1 expression constructs at increasing concentrations or empty vector pEF-BOS. After 24 h, cells were treated with or without 2ng/mL TGF-β for a further 24h before luciferase assay were conducted. Data were presented as the fold change as compared with the basal reporter level. **(B)** Ras reduces intracellular SPSB1 level. HEK 293T cells were transfected with indicated DNA constructs. After 48h, cells were lysed and analysed for the

expression of SPSB1, Ras and β-Actin. **(C)** Ras restores TGF-β signalling. HEK 293T cells were co-transfected with TGF-β reporter pCAGA-luc and indicated constructs for 24h. Thereafter, cells were treated with or without 2ng/mL TGF-β at indicated concentrations. After 24h, cells were lysed and luciferase activities were assessed as in Fig 1.9A. **(D)** Ras does not change the poly-ubiquitination of SPSB1. HEK 293T cells were co-transfected with constructs encoding FLAG -SPSB1 and indicated Ras expression constructs or empty vector pcDNA3 in the presence or absence of MYC-Ub expression constructs. After 48 h, cells were lysed and the cell lysates were incubated with FLAG beads for immunoprecipatation. Both cell lysates and immunoprecipatates were analysed for indicated proteins. **(E)** SPSB1 is detectable in exosomes from Ras-transformed cells. The SPSB-inducible Ras-transformed MDCK cells (21D1 cells) were cultured in serum free medium with or without 2μg/mL doxycycline for SPSB1 expression and exsome production. After 48h, exosomes were isolated, lysed and analysed for the expression of SPSB1, Ras, β-Actin and exosome markers TSG101 and Alix by Western blotting. **(F)** Proposed model. It is possible that exosomes are involved in the up-regulation of TGF-β signalling by mediating the discharge of cytosolic protein SPSB1 in the presence of Ras.

1.5 Hypothesis and aims

Exosomes are nanometre-sized vesicles (40-100 nm) secreted by various cells types. Exosomes contain a broad range of contents (such as DNA, RNA, proteins and lipids), exerting multiple biological effects. In particular, exosomes participate in signalling transduction by transferring signalling molecules.

TGF-β signalling, a key player in various cellular contexts, is tightly regulated by multiple molecules at each step of the signalling cascade. Dysregulation of TGF-β signalling causes the pathogenesis of human disease, including cancer. TGF-β can signal not only via linear signalling cascade but also through the crosstalk with the other pathways. Importantly, Ras signalling cooperates with TGF-β signalling in tumor progression. Ras enhances TGF-β signalling by suppressing the TβRII negative regulator, SPSB1. However, the mechanism by which Ras may decrease cellular SPSB1 levels is not clear because Ras does not affect the poly-ubiquitination of SPSB1.

Active TGF-β has been discovered in exosomes. Exosomal TGF-β is able to activate Smad-dependent signalling and drive fibroblast to myofibroblast differentiation. However, how exosomes participate in the regulation of TGF-β signalling is yet to be established. In particular, it is not clear how exosomal TGF-β may differ from free ligand TGF-β. In addition, SPSB1 (TβRII negative regulator) is detectable in exosomes from Ras-transformed cells. This observation raises the possibility that exosomes are involved in Ras-induced up-regulation of TGF-β signalling by exporting SPSB1 from the cell. This thesis addresses the **following hypotheses**:

(1) Exosomal TGF-β correlates with the metastatic potential of tumor cells, serving as a biomarker for metastatic cancer; exosomal TGF-β may function differently from free ligand TGF-β;

(2) Exosomes mediate the discharge of cytosolic TβRII negative regulator SPSB1 in a Ras-dependent manner;

(3) Ras-mediated oncogenic transformation results in the production of exosomal TGF-β.

The specific **aims** of this thesis are:

(1) To establish the relationship between the levels of exosomal TGF-β and the metastatic potential of tumor cell lines, and to examine how exosomal TGF-β may differ from free ligand TGF-β in activating TGF-β signalling;

(2) To establish a mechanism by which exosomes regulate intracellular protein levels: exosomes export cytosolic protein SPSB1 from the cell in the presence of Ras, thus reducing the cellular pool of SPSB1;

(3) To ascertain that Ras-induced transformation results in exosomal TGF-β production.

This thesis demonstrated that highly metastatic tumor cell lines displayed higher exosomal TGF-β levels as compared with non- /low- metastatic tumor cell lines, suggesting the potential of exosomal TGF-β as a biomarker for metastatic cancer. More importantly, exosomal TGF-β was able to restore TGF-β responsiveness in TGF-β receptor-defective cells. It will certainly lead to a re-evaluation of TGF-β signalling's contribution to the tumorigenesis of human colon cancer in which TβRIIs are mutationally inactivated. Ligand traps did not inhibit exosomal TGF-β-induced signalling efficiently. Consequently, strategies targeting exosomal TGF-β have to be developed.

The cytosolic protein SPSB1, a TβRII negative regulator, was secreted in association with exosomes upon Ras overexpression, causing a decrease in the cellular pool of SPSB1. Treatments using small molecular inhibitors suggested that Ca^{2+} concentration, ceramide, and PI3K were responsible for Ras-mediated SPSB1's MVB sorting and exosome secretion. SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain was required for SPSB1's recruitment to CD63-positive late endosome before they were released on exosomes. However, SPSB1's exosomal secretion was independent of the ubiquitination of SOCS box. Additionally, the uptake of SPSB1- and Ras- containing exosomes by recipient cells was observed. Taken together, these results highlighted the role of exosomes as vehicles for cytosolic protein clearance.

Lastly, this thesis showed that Ras overexpression resulted in exosomal TGF-β production, suggesting the crucial role of Ras in the release of exosomal TGF-β.

Chapter 2. Materials and Methods

2.1 DNA constructs, primers and virus

v-Ha-Ras was cloned into the pcDNA3 mammalian cell expression vector [256] and FLAG-SPSB1, MYC-SPSB1, MYC-SPSB1Δ, MYC-SPSB1(Y129A) were cloned into the pEF-BOS mammalian cell expression vector as previously described [257]. FLAG-TβRI, HA-TβRII were cloned into the pcDNA3 mammalian cell expression vector as previously described [258]. MYC-SPSB1 (K267R) was generated based on MYC-SPSB1 using Quick Change® II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's recommendations. Primers used for site-direct mutagenesis were: MYC-SPSB1 (K267R) forward: GAGGTAGGCTCTGAGGGAGGCAGGTAGCGG. MYC-SPSB1 (K267R) reverse: GAGGTAGGCTCTGAGGGAGGCAGGTAGCGG. All newly generated constructs were verified by sequencing.

The Ad-pCAGA-luciferase virus, Ad-CMV-Gaussia-luciferase virus [259] and Ad-CMV-Tomato virus [260] were produced and amplified as previously described. The Ad-CMV-sTβRII-Fc virus and Ad-CMV-Smad7 virus were produced according to protocol from Invitrogen. Briefly, the FLAG-Smad7 and sTβRII-Fc expression constructs were cloned into pENTR 1A entry clone vector. attL-arrR (LR) recombination was then performed with the *pAd/CMV/V5-DEST* destination vector (Invitrogen) to generate the pAd-CMV-sTβRII-Fc and pAd-CMV-Smad7 adenoviral expression plasmids. The expression plasmids were digested with Pac I to expose the inverted terminal repeats (ITRs) and then transfected into 293A cell line using Lipofectamine LTX transfection reagent (Invitrogen). After 2 weeks, cells were harvested when lysis was observed in majority of cells. The adenovirus were amplified, titred and used to infect cultured cells.

2.2 Antibodies and reagents

The following antibodies were used: anti-FLAG (M2) antibody (F3165, 1:5000 for WB and 1:2000 for IF) from Sigma-Aldrich; anti-cMYC antibody (sc-40, 1:1000 for WB) from Santa Cruz Biotechnology; anti-Ras antibody (610001, 1:1000 for WB and IF) from BD; anti-TSG101 antibody (612696, 1:1000 for WB) from BD; anti-Alix antibody (2127, 1:1000 for WB) from Cell Signalling; anti-CD81 antibody (sc-7637, 1:200 for WB) and anti-CD63 antibody (sc-5275, 1:500 for IF) from Santa Cruz Biotechnology; anti-Rab27a antibody (ab55667, 1:200 for WB) from Abcam; anti-tSmad2/3 antibody (610843,1:1000 for WB and

1:300 for IF) from BD; anti-β-Actin antibody (A2066, 1:5000 for WB) and anti-GAPDH antibody (G9545, 1:5000 for WB) from Sigma-Aldrich; anti-TβRI (V-22, 1:500 for WB) and anti-TβRII (L-21, 1:1000 for WB) from Santa Cruz Biotechnology; anti-hIgG1-Fc antibody (MAB110, 1:1000 for WB) and anti-ecTβRII (AF-1003, 1:500 for WB) from R&D systems. Rabbit polyclonal anti-SPSB1 antibody (1:500 for IF) was generated in house; rabbit polyclonal anti-phospho-Smad2 antibody (1:1000 for WB) was a gift from Prof. Peter ten Dijke (Leiden University Medical Center, Netherlands). HRP-conjugated secondary antibodies (1:1000) were purchased from Bio-Rad. The Alexa Fluor® 488- and Alexa Fluor® 546-conjugated secondary antibodies (1:500) used for immunofluorescence staining were purchased from Invitrogen.

Hoechst (H6024) was purchased from Sigma-Aldrich. Monensin (M5273), wortmannin (W1628), GW4869 (D1692), Brefeldin A (B7651), 5-(N,N-Dimethyl) amiloride hydrochloride (DMA) (A4562) and SB431542 (S4317) were purchased from Sigma-Aldrich. Human recombinant TGF-β1 were purchased from R&D Systems. Control siRNA-A (sc-37007) and Rab27a siRNA (sc-41834) were purchased from Santa Cruz Biotechnology.

2.3 Cell lines, cell culture and transfection

Human breast cancer cell lines MCF-7 and MDA-MB-231, the Madin-Darby canine kidney (MDCK) cells and v-Ha-Ras-transformed MDCK (21D1) cells [119], mouse fibroblast cell line NIH3T3 and v-Ha-Ras-transformed NIH3T3 cell line (Ras-NIH3T3) [261], human embryonic kidney cell line HEK 293T , human colon cancer cell line T84, mutant mink lung epithelial (Mv1Lu) cell lines R1B and DR26 were cultured in DMEM (Gibco, Life technology). Human colon cancer cell lines SW480 and SW620 were maintained in RPMI 1640 (Gibco, Life technology). Human bladder cancer cell lines RT4 and T24 were cultured in McCoy's 5a (Gibco, Life technology). All cultured cells were supplemented with 10% fetal calf serum, 10 μg/mL penicillin and 100 μg/mL streptomycin (Invitrogen) at 37 °C with 10% CO_2 in a humidified atmosphere. HEK293T, MDA-MB-231, MCF-7 and NIH3T3 were originally obtained from ATCC (American Type Culture Collection). MDCK and 21D1, Ras-NIH3T3, SW480 and SW620 were gifts from Prof. Richard J. Simpson (La Trobe Institute for Molecular Science (LIMS), La Trobe University). T84 was a gift from Dr. Oliver M. Sieber (Walter and Eliza Hall Institute of Medical Research (WEHI), University of Melbourne). T24 and RT4 were gifts from A/Prof Chris Hovens (Department of Surgery,

University of Melbourne). R1B and DR26 were gifts from A. B. Roberts (National Institutes of Health).

HEK 293T cells were transiently transfected with indicated DNA constructs using FuGENE HD transfection reagent (Promega). MDA-MB-231 cells were transiently transfected with Control siRNA-A or Rab27a siRNA using Lipofectamine® RNAiMAX Reagent (Life Technologies) according to the manufacturer's protocol.

2.4 Exosome isolation

Exosomes were isolated by differential centrifugation as previously described with slight modifications [262]. Briefly, HEK 293T cells (4×10^5-5×10^7) transfected with indicated DNA constructs were grown for 48h before cells were cultured in bovine exosomes-depleted medium (DMEM containing 1% FCS and 1% penicillin-streptomycin was centrifuged overnight at 100,000 g to deplete bovine exosomes) or serum free medium for 24h. Other cell lines (1×10^7-4×10^8) were cultured in serum free medium for 24h. The culture media were harvested and subjected to sequential centrifugation steps (300×g for 5min, 2000×g for 15 min). The supernatants were further concentrated by 100K NMWL centrifugal filtration (Amicon Ultra-15, Millipore) and washed twice with 1 × PBS before centrifuged at 10,000×g for 90min. Exosomes were recovered from the cleared, condensed supernatant by ultracentrifugation at 100,000 × g for 17h at 4 °C. Exosome pellet was resuspended in ice-cold 1 × PBS and stored at 4°C. Exosomes used for Western blotting analysis were resuspended in 1×Laemmli Sample Buffer (Bio-Rad) before application. Exosomal protein amount was determined by BCA Protein Assay Kit (Thermo Scientific).

2.5 Cryo-TEM analysis

Cryo-transmission electron microscopy (cryo-TEM) analysis of exosomes was performed as previously described [119]. Briefly, isolated exosomes were resuspended in ice-cold 1× PBS and transferred onto glow-discharged C-flat holey carbon grids. Excess liquid was blotted and grids were frozen and mounted in a Gatan cryoholder in liquid nitrogen. The grids were transferred to Tecnai F30 (FEI) electron microscope at liquid nitrogen temperature and the samples were observed at 300 kV. Representative images were presented.

2.6 Western blotting analysis

Cells were lysed using lysis buffer (30mM HEPES, 1% TritonX-100, 2mM MgCl, 150mM NaCl, 5mM EDTA, complete protease inhibitor tablet and phosphostop phosphatase cocktail tablet) on ice for 30min. The cell debris was removed by centrifugation at 13000 rpm for 15 min. The lysate was heated at 95 °C for 10 minutes in 1 × Laemmli sample buffer (Bio-Rad). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare). After blocking with 4% skim milk for 1h at room temperature (RT), the membranes were probed with indicated primary antibody and corresponding secondary antibody. The signal was visualized by using the Western Lightning® ECL Pro Enhanced Chemiluminescence Substrate (PerkinElmer). All Western blotting results were representative of three separate experiments.

2.7 Luciferase assay

Reporter cells (3000 cells/well in 96-well plate) were seeded and co-infected with Ad-pCAGA-luciferase virus (MOI: 5000) and Ad-CMV-Gaussia-luciferase virus (MOI: 1000) as previously described [259]. After 24h infection, reporter cells were stimulated with indicated samples (free-ligand TGF-β or exosomes) for further 24h. Thereafter, cells were lysed and assessed for luciferase activity using Luciferase Assay Kit (Promega) following the manufacturer's instructions. Luciferase activities (TGF-β reporter activities) were presented as the fold change as compared with the basal reporter level. The exosomal TGF-β level was determined using standard curve generated by plotting the various concentrations of free-ligand TGF-β against the measured TGF-β reporter activities. Data shown were means of triplicates ± SD and were representative of three separate experiments.

2.8 Immunofluorescence staining and confocal microscopy

HEK 293T cells (4×10^5) were grown on glass cover slips and transfected with indicated DNA constructs for 48h. MDA-MB-231 cells or NIH3T3 fibroblasts were seeded on an 8-well chamber slide (6×10^3 cells/well) (Thermo Scientific) for 24h and stimulated with 10μg/mL exosomes or indicated concentrations of free-ligand TGF-β for 1h. Cells were fixed in 3.7% formaldehyde in PBS (Sigma-Aldrich) for 10 min at RT. After washing 3 times with 1×PBS, cells were permeabilized with 0.1% Triton-X-100 (Merck) for 5 min at RT and washed 3 times with 1× PBS. Cells were then blocked with PBS containing 5% BSA for 1 h at RT. Thereafter, cells were probed with indicated primary antibodies for 2h at RT. After washing

with 1× PBS for 3 times, cells were incubated with Alexa Fluor® 488- or Alexa Fluor® 546-conjugated secondary antibodies for 1h at RT and were then washed 3 times with 1× PBS. Cell nuclei were stained with Hoechst stain for 10 minutes at RT. Following three times 1× PBS wash and one time double-distilled water (DDW) wash, HEK 293T cells were mounted onto a glass slide while MDA-MB-231 cells and NIH3T3 fibroblasts were mounted onto glass cover slips using Prolong Gold antifade reagent (Invitrogen). Images were collected by Olympus FV1000 confocal microscope (magnification = 40× or 60×) and processed using Olympus Fluroview Ver. 1.7c. Representative images were presented.

2.9 Statistics

All statistical analyses were calculated by Student's t -test. Error bars indicate s.d (standard deviation).

Chapter 3.

Cancer exosomes transduce receptor-independent TGF-β signalling and evade ligand trapping therapy

Cancer exosomes transduce receptor-independent TGF-β signalling and evade ligand trapping therapy

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Abstract

Exosomes are well recognized as carriers of signalling molecules, including transforming growth factor-β (TGF-β), in intercellular communications. It is not clear whether and how exosomal TGF-β may differ from the free-ligand TGF-β in transducing signalling. We report for the first time that exosomal TGF-β restores TGF-β signalling in cells defective of its functional receptors i.e. without re-expression of the receptors. In addition, ligand trapping therapies such as soluble type II receptor-Fc (sTβRII-Fc) are inefficient in blocking exosomal TGF-β. Furthermore, free-ligand TGF-β treatment increases exosomal TGF-β secretion which is produced highly in metastatic cancer cell lines. Our data shed new lights on the tumorigenesis of cancers with loss-of-function of TβRII and the defects of ligand-trapping therapies as well as point to the potentials for exosomal TGF-β to be a biomarker for cancer progression and a therapeutic target.

Introduction

Exosomes are bioactive small membrane vesicles (40-100nm in diameter) secreted by various cell types [1]. They are generated from late endosomes/multivesicular bodies (MVBs) [2] and are released into the extracellular space upon the fusion of MVBs outer membrane with the plasma membrane [3]. Exosomes participate in a broad range of biological processes by carrying various contents, such as proteins, DNAs, RNAs and lipids [4]. In particular, exosomal transfer of oncogenic cargoes, such as Ras [5], EGFRVIII [6] and LMP1 [7] , has been linked to enhanced tumor aggressiveness [8, 9]. As such, exosomes are proposed as a potential biomarker for cancer progression [10, 11]. Yet, little practical progress has been made towards this end. Transforming growth factor-β (TGF-β) plays a key role in cancer development and has been discovered in exosomes [12]. Thus, exosomal TGF-β appear to be an ideal candidate as a biomarker for cancer progression. On the other hand, it is beginning to emerge that exosomal TGF-β may differ from free-ligand TGF-β [13]. However, little is known about the exact nature of the differences between exosomal TGF-β and free-ligand TGF-β. Identification of these differences may present unprecedented opportunities for advancing our understanding of tumorigenesis and for discovering novel strategies against cancer.

Results

Cancer exosomes activates TGF-β-Smad signalling.

Previous studies have revealed the presence of active TGF-β in exosomes secreted by a diverse range of cancer cells [14]. To test whether this is applicable to MDA-MB-231 cells, a highly metastatic human breast cancer cell line, we isolated and characterized exosomes from the culture media of MDA-MB-231 cells using a differential centrifugation protocol [15] (Fig. 1A). Notably, Retentate I was washed twice with 1× PBS and concentrated using 100K NMWL centrifugal units to remove free-ligand TGF-β. Cryo-transmission electron microscopy (cryo-TEM) analysis showed that P100 fraction displayed exosome-typical size and morphology. Immunoblotting confirmed the presence of exosome markers (TSG101 and Alix) in P100 fraction (Fig. 1B), confirming P100 as exosomes.

We next investigated whether exosomes released by MDA-MB-231 cells (MDA exosomes) have the capacity to induce TGF-β signalling activity. The activation of TGF-β signalling was observed after addition of 10,000 × g supernatant (supernatant III in Fig. 1A) as measured by TGF-β reporter activity assay using the reporter adenovirus infected 21D1 cells [16]. In comparison, the TGF-β signalling-inducing activity were completely depleted in 100,000 × g supernatant (supernatant IV in Fig. 1A) after exosomes were pelleted by ultracentrifugation (Supplementary Information, Fig. S1A), indicating that active TGF-β were in association with MDA exosomes. This result also suggested that free-ligand TGF-β was cleared during differential centrifugation steps. As expected, addition of MDA exosomes resulted in strong activation of TGF-β signalling, similar to that induced by free-ligand TGF-β (Fig. 1C; Supplementary Information, Fig. S1B).

Moreover, we examined receptor-regulated Smads (R-Smads) responses to active TGF-β-containing MDA exosomes. The activation of TGF-β signalling leads to phosphorylation of R-Smads which subsequently form heterotrimeric complexes with Smad4 and translocate into the nucleus where they activate or suppress the transcription of target genes by associating with transcriptional factors [17-19]. The Smad3-responsive TGF-β reporter activity assay (Fig. 1C) confirmed the activation of Smad3-mediated TGF-β signalling by MDA exosomes. Consistently, the phosphorylation and nuclear translocation of Smad2 were also observed in NIH3T3 fibroblasts (Fig. 1D, E) and in MDA-MB-231 cells (Supplementary Information, Fig. S1C, D) when treated with MDA exosomes or with the free-ligand TGF-β but not in the

untreated controls. Taken together, these results demonstrate that MDA-MB-231 cells produce TGF-β-positive exosomes which activate TGF-β-Smad signalling.

To further confirm that active TGF-β is released in association with exosomes, Rab27a, a key regulator in exosome secretion [20-22], was targeted by siRNA for knockdown (Fig. 1F). Indeed, Rab27a knockdown resulted in a marked reduction in the exosome production as demonstrated by decreased exosome marker TSG101 and Alix (Supplementary Information, Fig. S1E). Consequently, a significant reduction in exosomal TGF-β activity was observed in Rab27a knockdown cells as compared with control cells (Fig. 1F). Thus, Rab27a is required for the release of exosomal TGF-β.

Highly metastatic tumor cells produce higher levels of exosomal TGF-β than low metastatic tumor cells.

The levels of active TGF-β in MDA-MB-231-produced exosomes (70pg/μg exosomes) were much higher than those reported of MCF-7 cells (3pg/μg exosomes, low metastatic human breast cancer cell line) [14]. Is there a correlation between the levels of tumor-derived exosomal TGF-β and the metastatic potential of tumor cells? The levels of exosomal TGF-β from the "normal" human embryonic kidney 293T (HEK 293T) cells and three pairs of low-/high- metastatic human cancer cell lines (breast cancer MCF-7 and MDA-MB-231; colon cancer SW480 and SW620; bladder cancer cell lines RT4 (non-metastatic) and T24) were examined using the TGF-β reporter activity assay. The exosomes from HEK 293T cells showed undetectable exosomal TGF-β. Each of the highly metastatic cancer cell lines (MDA-MB-231, SW620 and T24) exhibited much higher levels of exosomal TGF-β than their low-/non- metastatic counterparts (MCF-7, SW480 and TR4) on the basis of the same number of cells (Fig. 2A). In fact, it was also true on the basis of the same amount of exosomes (Fig. 2B; Supplementary Information, Fig. S2A). As such, the exosomal TGF-β has the potential to be a biomarker for cancer progression.

Cancer cells are physiologically exposed to active TGF-β in tumor microenvironment *in vivo* [23]. We then evaluated the effect of free-ligand TGF-β on the levels of exosomal TGF-β. Exosomes were harvested from MDA-MB-231 cells and MCF-7 cells treated with or without 5ng/mL free-ligand TGF-β. To rule out the contamination of free-ligand TGF-β in exosome fraction, Retentate I (Fig. 1A) were washed with 1× PBS for three times during concentration using 100K NMWL centrifugal units. The 100,000 × g supernatant showed no TGF-β activity (Supplementary Information, Fig. S2E), indicating that free-ligand TGF-β was cleared from the exosome fraction. Free-ligand TGF-β treatment resulted in an elevation in the levels of exosomal TGF-β in both MCF-7 and MDA-MB-231 cells (Fig. 2C). While more active TGF-β were loaded into exosomes upon TGF-β stimulation (Fig. 2D), the exosome production was not affected (Fig. 2E). It has been shown that the exosomal packaging of ErbB1, a member of EGFR family, is enhanced upon EGF stimulation, suggesting the involvement of ligand-dependent ErbB1 activation in the release of exosomal ErbB1 [24]. Given that active TGF-β ligand-receptor complexes undergo endocytosis and are targeted to endosomal trafficking [25, 26] where the exosomes are generated, it is possible that the activation of TGF-β ligand-receptor complexes contributes to the production of exosomal TGF-β.

Exosomal TGF-β restores TGF-β responsiveness in TGF-β receptor-defective cells.

It has been previously shown that exosomal TGF-β rather than free-ligand TGF-β drive bone-marrow mesenchymal stem cell (BM-MSC) to myofibroblast differentiation [13], suggesting differential function between exosomes and their cargoes. To investigate the differences, we assessed TGF-β signalling in the TGF-β type I receptor (TβRI)-defective R1B, the TGF-β type II receptor (TβRII)-defective DR26 [27] and the Smad4-defective T84 cells [28]. Consistent with previous reports, the free-ligand TGF-β failed to activate TGF-β signalling in these cells. Surprisingly, MDA-MB-231 cells-produced exosomes transduced TGF-β signalling in R1B and DR26 cells but not in T84 cells (Fig. 3A). The restoration of TGF-β responsiveness in either R1B or DR26 cells requires the expression of TβRI and TβRII, respectively [29]. It is possible that TβRI and TβRII, but not Smad4, are carried in the TGF-β-positive exosomes.

To determine whether TβRI and TβRII are released in association with exosomes, we transfected HEK 293T cells with TβRI and TβRII expression plasmids. Immunoblotting analysis of the exosomes from transfected cells demonstrated the presence of TβRI and TβRII in exosomes (Fig. 3B). Interestingly, while multiple TβRII bands (due to different glycosylation status [30]) were detected in cell lysates, TβRII was detected as a single band in exosomes. This observation suggested that the sorting of TβRII into exosomes might depend on the glycosylation status of TβRII. Collectively, we conclude that TGF-β-positive exosomes contain the TβRIs and TβRIIs, and transduce TGF-β signalling in TGF-β receptor-defective cells.

Inactivation of TβRIIs has been observed in a subset of human colon cancers (15% of sporadic ones) and it was believed that these cancer cells lost TGF-β responsiveness [31, 32]. However, our finding that exosomal TGF-β transduces TGF-β signalling in TGF-β receptor-defective cells raises the possibility that cancer cells lacking functional TGF-β receptors may re-activate TGF-β signalling by receiving exosomes derived from the tumor microenvironment. Human carcinomas are surrounded by stromal fibroblasts, also known as carcinoma-associated fibroblasts (CAFs), which promote tumor growth by regulating paracrine signalling [33, 34]. To mimic the tumor stroma, R1B, DR26 and T84 cells were co-cultured with NIH3T3 fibroblasts, which produced TGF-β-positive exosomes (Supplementary Information, Fig. S3A). Indeed, TGF-β responsiveness was restored in both R1B and DR26 cells when co-cultured with NIH3T3 fibroblasts, which was enhanced upon

free-ligand TGF-β treatment (Fig. 3C,D). In contrast, no TGF-β signalling activation was observed in T84 cells when co-cultured with NIH3T3 fibroblasts (Fig. 3E). These results suggest the possibility that TGF-β signalling in receptor-defective cells is restored by TGF-β receptors-containing exosomes derived from NIH3T3 fibroblasts. Thus, human colon cancers once thought of defective of TGF-β signalling due to inactivation of TβRII may be activated by tumor stroma-derived exosomes. Consequently, TGF-β-driven tumorigenesis, particularly in colon cancer progression, deserves to be carefully re-examined.

Inefficient inhibition of exosomal TGF-β-induced signalling by ligand trapping therapies.

Several types of ligand trapping therapies have been developed, such as soluble TGF-β receptor II-Fc (sTβRII-Fc) [35] and TGF-β neutralizing antibodies [36]. Given that exosomal TGF-β differ from the free-ligand TGF-β in transducing signalling (Fig. 3A), we investigated whether ligand trapping therapies block the exosomal TGF-β as efficiently as the free-ligand TGF-β. We used adenovirus to successfully express sTβRII-Fc which was confirmed by immunoblotting (Fig. 4A) and Coomassie Blue Staining (Supplementary Information, Fig. S3B). As shown in Fig. 4B-C, sTβRII-Fc inhibited 1ng/mL and 2ng/mL free-ligand TGF-β-induced signalling activity by 78% and 62%, respectively. However, sTβRII-Fc was less efficient in blocking TGF-β signalling induced by exosomal TGF-β. The sTβRII-Fc treatment only reduced exosomes-induced TGF-β signalling activity by around 20% or less. In addition, the levels of free-ligand TGF-β and exosomal TGF-β were measured by TGF-β reporter activity and TGF-β1 ELISA assays. The levels of free-ligand TGF-β detected by TGF-β reporter assay were the same as using ELISA assay. However, less than 50% of exosomal TGF-β were detected by ELISA when compared with using TGF-β reporter assay (Fig. 4D). Taken together, these results suggest that the exosomal TGF-β may not be readily accessible to sTβRII-Fc or TGF-β antibodies, possibly due to the ligand-receptor complex formation on the exosomes. We then examined the inhibitory effects of SB431542 [37] and Smad7 on TGF-β signalling induced either by the free-ligand TGF-β or the exosomes. SB431542 is a small-molecule inhibitor that inhibits TβRI kinase activity, while Smad7 blocks phosphorylation of R-Smads [38] and suppresses the binding of Smad4/R-Smads complex to target gene [39]. As shown in Fig. 4E-H, either treatment resulted in significant inhibitions of free-ligand TGF-β-induced signalling and a comparable inhibition of exosomes-induced signalling. As such, ligand trapping therapies targeting free-ligand TGF-β may not be applicable to exosomal TGF-β, although strategies targeting intracellular components of TGF-β signalling work for both free-ligand TGF-β- and exosomal TGF-β-induced signalling.

Discussion

Current study first demonstrate that TGF-β-positive exosomes contain TGF-β ligands and receptors (TβRIs and TβRIIs) with an unexpected biological property of restoring TGF-β responsiveness in TGF-β receptor-defective cells, strongly suggesting a reconsideration of tumorigenesis in cancers with the receptor loss-of-function mutation. Therapeutically, ligand-trapping therapies (sTβRII-Fc) do not inhibit exosomal TGF-β-induced signalling efficiently, implying the requirement for a novel ligand targeting strategy. In addition, we identify that highly metastatic human cancer cell lines produce higher levels of exosomal TGF-β as compared with low-/non- metastatic human cancer cell lines, indicating the potential of exosomal TGF-β as a novel biomarker for cancer progression.

Our data showed that active TGF-β were enriched in exosomes released from human cancer cells, particularly from the highly metastatic cells, but not from "normal" human HEK 293T cells (Fig. 2A, B).The production of exosomal TGF-β was further enhanced by the free-ligand TGF-β treatment (Fig. 2C-E), which is physiologically relevant since cancer cells are exposed to circulating TGF-β *in vivo*. Given the role of TGF-β in cancer progression, the differential exosomal TGF-β levels between highly metastatic cancer cells and low metastatic cells (Fig. 2A, B) imply that the levels of exosomal TGF-β are reflective of the metastatic potential of tumor cells. Because metastasis is the major cause of cancer-related death [40], identifying novel biomarkers for metastasis has become the focus of much research. Our finding suggests a great diagnostic and prognosis potential of exosomal TGF-β as a biomarker for metastatic cancer. Given the fact that exosome-based diagnostic assays are currently unavailable in clinic due to the time-consuming and labour intensive isolation procedure, more sensitive, quicker methods should be developed for the detection of exosomal TGF-β.

In general, the transduction of TGF-β signalling requires the formation of ligand-receptor complex and the participation of Smad proteins [41]. As such, free-ligand TGF-β didn't activate TGF-β signalling in either TGF-β receptor-defective or Smad4-defective cells [27, 42]. Surprisingly, exosomal TGF-β restored TGF-β responsiveness in TGF-β receptor-defective cells (R1B and DR26) but not in Smad4-defecitve cells (Fig. 3A), indicating that TGF-β receptors rather than Smad4 are carried in exosomes. Indeed, immunoblotting confirmed the presence of TβRI and TβII in exosomes (Fig. 3B). Active TGF-β ligand-receptor complexes have been shown to undergo endocytosis and been targeted to endosomal

trafficking [26, 43]. Because exosomes originate from endosomes, activation of ligand-receptor complex is likely to be involved in the production of TGF-β ligand- and receptors-containing exosomes. Supporting this notion, free-ligand TGF-β treatment resulted in increased exosomal TGF-β production (Fig. 2C).

Human carcinomas are surrounded by stromal fibroblasts, also known as carcinoma-associated fibroblasts (CAFs), which promote tumor growth by regulating paracrine signalling [33, 34]. NIH3T3 fibroblasts secreted TGF-β-positive exosomes (Supplementary Information, Fig. S3A). Activation of TGF-β signalling (which was further enhanced by free-ligand TGF-β treatment) was observed in TGF-β receptor-defective cells when they were co-cultured with NIH3T3 fibroblasts (Fig. 3C,D). CAFs-derived exosomes have been implicated in angiogenesis, invasion and metastasis [44] by activating multiple signalling pathways. Our result suggests the possible role of CAFs-derived exosomes in TGF-β signalling transduction and tumorigenesis of TGF-β receptor-defective cells. The mutational inactivation of TβRII is commonly observed in human colorectal cancer, with more than 90% occurrence in microsatellite instable (MSI) patients [31, 32]. It was believed that TGF-β signalling in those tumors was impaired because of the inactivating mutation of TβRIIs. However, our observation that TβRI and TβRII are secreted in association with exosomes raises the possibility that TGF-β signalling in those colon cancer patients may be restored by TβRIs- and TβIIs-containing exosomes derived from tumor stroma. Further experiments will be required to test the impact of exosomes on the TGF-β signalling in TβRII-defective colon cancer development.

Because of the promoting role of TGF-β signalling in tumor progression, several strategies (such as TGF-β neutralizing antibodies, sTβRII-Fc and small molecular inhibitors) that target different components of TGF-β signalling pathway have been developed and shown efficacy [36]. However, our data demonstrate that exosomal TGF-β can evade ligand-trapping therapies. The sTβRII-Fc sequesters TGF-β ligands to block TGF-β signalling [35]. Although sTβRII-Fc treatments significantly suppressed free-ligand TGF-β-induced signalling, they failed to efficiently inhibit TGF-β signalling caused by exosomal TGF-β (Fig.4A-C). In addition, TGF-β ELISA assay only detected less than 50% of exosomal TGF-β level than that detected by TGF-β reporter activity assay (Fig. 4D). Given that ELISA assay is based on the capture and detection of free molecules by antibodies, our results indicate that the exosomal transport of active TGF-β may block the access of TGF-β antibodies to TGF-β ligand carried in exosomes. In contrast, SB431542 and Smad7 inhibited exosomes-induced signalling as

efficiently as the free-ligand TGF-β-induced signalling (Fig. 4E-H). SB431542 is a small-molecule inhibitor that inhibits TβRI kinase activity, while Smad7 blocks phosphorylation of R-Smads [38] and suppresses the binding of Smad4/R-Smads complex to target gene [39]. These observations indicate that exosomal TGF-β-induced and free-ligand TGF-β-induced signalling differ at ligand-receptor level, but not in the intracellular signalling transduction. Because TGF-β ligand-receptor complexes have been shown to undergo endocytosis and been targeted to endosomal trafficking (where exosomes are generated), it is possible that TGF-β ligands and receptors already form complexes in exosomes. As such, TGF-β antibodies and ligand trapping reagents cannot gain direct access to TGF-β ligands carried in exosomes, causing ineffective blocking and detection. Our results suggest that strategies targeting exosomal TGF-β need to be developed to effectively block TGF-β signalling initiation.

In conclusion, our study discovers functional and therapeutic differences between free-ligand TGF-β and exosomal TGF-β. Specifically, exosomal TGF-β has the capacity of signalling in TGF-β receptor-defective cells. This finding has important impact on our understanding of the tumorigenesis of human colon cancers with TβRII defective mutation. Therapeutically, exosomal TGF-β can circumvent the efficacy of ligand-trapping reagents. Taken together, exosomal TGF-β has the potential to be a biomarker for cancer progression. More broadly, the specific findings here with TGF-β may be applicable to other growth factors/cytokines-receptors. A new chapter for exosomal growth factor signalling in tumorigenesis and therapeutic approach may begin.

Material and Methods

DNA constructs and adenovirus

FLAG-TβRI, HA-TβRII were cloned into the pcDNA3 mammalian cell expression vector as previously described [27]. The Ad-pCAGA-luciferase virus, Ad-CMV-Gaussia-luciferase virus [45] and Ad-CMV-Tomato virus [16] were produced and amplified as previously described. The Ad-CMV-sTβRII-Fc virus and Ad-CMV-Smad7 virus were generated according to protocol from Invitrogen. Briefly, the FLAG-Smad7 and sTβRII-Fc expression constructs were cloned into pENTR 1A entry clone vector. attL-arrR (LR) recombination was then performed with the *pAd/CMV/V5-DEST* destination vector (Invitrogen) to generate the pAd-CMV-sTβRII-Fc and pAd-CMV-Smad7 adenoviral expression plasmids. The expression plasmids were digested with Pac I to expose the inverted terminal repeats (ITRs) and then transfected into 293A cell line using Lipofectamine LTX transfection reagent (Invitrogen). After 2 weeks, cells were harvested when lysis was observed in majority of cells. The adenovirus were amplified, titred and used to infect cultured cells.

Antibodies and reagents

The following antibodies were used: anti-TSG101 antibody (612696, 1:1000 for WB) from BD; anti-Alix antibody (2127, 1:1000 for WB) from Cell Signalling; anti-Rab27a antibody (ab55667, 1:200 for WB) from Abcam; anti-tSmad2/3 antibody (610843,1:1000 for WB and 1:300 for IF) from BD; anti-β-Actin antibody (A2066, 1:5000 for WB), and anti-GAPDH antibody (G9545, 1:5000 for WB) from Sigma-Aldrich; anti-TβRI (V-22, 1:500 for WB) and anti-TβRII (L-21, 1:1000 for WB) from Santa Cruz Biotechnology; anti-hIgG1-Fc antibody (MAB110, 1:1000 for WB) and anti-ecTβRII (AF-1003, 1:500 for WB) from R&D systems; anti-FLAG (M2) antibody (F3165, 1:5000 for WB) from Sigma-Aldrich; rabbit polyclonal anti-phospho-Smad2 antibody (1:1000 for WB) was a gift from Prof. Peter ten Dijke (Leiden University Medical Center, Netherlands). HRP-conjugated secondary antibodies (1:1000) were purchased from Bio-Rad. The Alexa Fluor® 488- and Alexa Fluor® 546-conjugated secondary antibodies (1:500) used for immunofluorescence staining were purchased from Invitrogen.

Hoechst (H6024) was purchased from Sigma-Aldrich. Control siRNA-A (sc-37007) and Rab27a siRNA (sc-41834) were purchased from Santa Cruz Biotechnology. Human

recombinant TGF-β1 was purchased from R&D Systems. SB431542 (S4317) was purchased from Sigma-Aldrich.

Cell lines, cell culture and transfection

Human breast cancer cell lines MCF-7 and MDA-MB-231, the v-Ha-Ras-transformed Madin-Darby canine kidney (MDCK) cells (21D1 cells) [46], mouse fibroblast cell line NIH3T3, human embryonic kidney cell line HEK 293T , human colon cancer cell line T84, mutant mink lung epithelial (Mv1Lu) cell lines R1B and DR26 were cultured in DMEM (Gibco, Life technology). Human colon cancer cell lines SW480 and SW620 were maintained in RPMI 1640 (Gibco, Life technology). Human bladder cancer cell lines RT4 and T24 were cultured in McCoy's 5a (Gibco, Life technology). All cultured cells were supplemented with 10% fetal calf serum, 10 μg/mL penicillin and 100 μg/mL streptomycin (Invitrogen) at 37 °C with 10% CO_2 in a humidified atmosphere. HEK293T, MDA-MB-231, MCF-7 and NIH3T3 were originally obtained from ATCC (American Type Culture Collection). 21D1, SW480 and SW620 were gifts from Prof. Richard J. Simpson (La Trobe Institute for Molecular Science (LIMS), La Trobe University). T84 was a gift from Dr. Oliver M. Sieber (Walter and Eliza Hall Institute of Medical Research (WEHI), University of Melbourne). T24 and RT4 were gifts from A/Prof Chris Hovens (Department of Surgery, University of Melbourne). R1B and DR26 were gifts from A. B. Roberts (National Institutes of Health).

HEK 293T cells were transiently transfected with indicated DNA constructs using FuGENE HD transfection reagent (Promega). MDA-MB-231 cells were transiently transfected with Control siRNA-A or Rab27a siRNA using Lipofectamine® RNAiMAX Reagent (Life Technologies) according to the manufacturer's protocol.

Exosome isolation

Exosomes were isolated by differential centrifugation as previously described with slight modifications [15]. In Fig. 2A, B and Fig. S2A, exosome were harvested from the following cell lines: HEK 293T (1.65×10^7), MCF-7 and MDA-MB-231 (1×10^8), SW480 and SW620 (4×10^8), RT4 and T24 (5×10^7). Briefly, the culture media were harvested and subjected to sequential centrifugation steps (300 × g for 5min, 2000 × g for 15 min). The supernatants were further concentrated by 100K NMWL centrifugal filtration (Amicon Ultra-15, Millipore) and washed twice (three times for free-ligand TGF-β-treated cells shown in Fig.

2C-Fig. 2E) with 1× PBS before centrifuged at 10,000 × g for 90min. Exosomes were recovered from the cleared, condensed supernatant by ultracentrifugation at 100,000 × g for 17h at 4 °C. Exosome pellet was resuspended in ice-cold 1 × PBS and stored at 4°C. Exosomes used for Western blotting analysis were resuspended in 1× Laemmli Sample Buffer (Bio-Rad) before application. The concentration of exosomal proteins was quantified by BCA Protein Assay Kit (Thermo Scientific).

Cryo-TEM analysis

Cryo-transmission electron microscopy (cryo-TEM) analysis of exosomes was performed as previously described [46]. Briefly, isolated exosomes were resuspended in ice-cold 1× PBS and transferred onto glow-discharged C-flat holey carbon grids. Excess liquid was blotted and grids were frozen and mounted in a Gatan cryoholder in liquid nitrogen. The grids were transferred to Tecnai F30 (FEI) electron microscope at liquid nitrogen temperature and the samples were observed at 300 kV. Representative images were presented.

Western blotting analysis

Cells were lysed using lysis buffer (30mM HEPES, 1% TritonX-100, 2mM MgCl, 150mM NaCl, 5mM EDTA, complete protease inhibitor tablet and phosphostop phosphatase cocktail tablet) on ice for 30min. The cell debris was removed by centrifugation at 13000 rpm for 15 min. The lysate was heated at 95 °C for 10 minutes in 1 × Laemmli sample buffer (Bio-Rad). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare). After blocking with 4% skim milk for 1h at room temperature (RT), the membranes were probed with indicated primary antibody and corresponding secondary antibody. The signal was visualized by using the Western Lightning® ECL Pro Enhanced Chemiluminescence Substrate (PerkinElmer). All Western blotting results were representative of three separate experiments.

Luciferase assay

Reporter cells (3000 cells/well in 96-well plate) were seeded and co-infected with Ad-pCAGA-luciferase virus (MOI: 5000) and Ad-CMV-Gaussia-luciferase virus (MOI: 1000) as previously described [45]. After 24h infection, reporter cells were stimulated with indicated samples (free-ligand TGF-β or exosomes) for further 24h. Thereafter, cells were lysed and assessed for luciferase activity using Luciferase Assay Kit (Promega) following the manufacturer's instructions. Luciferase activities (TGF-β reporter activities) were presented

as the fold change as compared with the basal reporter level. The exosomal TGF-β level was determined using standard curve generated by plotting the various concentrations of free-ligand TGF-β against the measured TGF-β reporter activities. Data shown were means of triplicates ± SD and were representative of three separate experiments.

Immunofluorescence staining and confocal microscopy

MDA-MB-231 cells or NIH3T3 fibroblasts were seeded on an 8-well chamber slide (6×10^3 cells/well) (Thermo Scientific) for 24h and stimulated with 10μg/mL exosomes or indicated concentrations of free-ligand TGF-β for 1h. Cells were washed 3 times with 1×PBS and fixed in 3.7% formaldehyde in PBS (Sigma-Aldrich) for 10 min at RT. After washing 3 times with 1×PBS, cells were permeabilized with 0.1% Triton-X-100 (Merck) for 5 min at RT and washed 3 times with 1× PBS. Cells were then blocked with PBS containing 5% BSA for 1 h at RT. Thereafter, cells were probed with tSmad2/3 antibody (Mouse, 1:300) for 2h at RT. After washing with 1× PBS for 3 times, cells were incubated with Alexa Fluor® 488 goat anti-mouse IgG (1:500) for 1h at RT and were then washed 3 times with 1× PBS. Cell nuclei were stained with Hoechst stain for 10 minutes at RT. Following three times 1× PBS wash and one time double-distilled water (DDW) wash, MDA-MB-231 cells and NIH3T3 fibroblasts were mounted onto glass cover slips using Prolong Gold antifade reagent (Invitrogen). Images were collected by Olympus FV1000 confocal microscope (magnification = 40×) and processed using Olympus Fluroview Ver. 1.7c. Representative images were presented.

TGF-β1 ELISA Assay

Free-ligand TGF-β and exosomal TGF-β were quantified using Human TGF-β1 Quantikine ELISA kit (R&D systems) according to manufacturer's instructions. In short, 50uL of Assay diluent RD1-21 were added to each well. Thereafter, 50uL of Free-ligand TGF-β standard or exosomes (3μg/mL) were added, mixed, and incubated for 2h at RT. After washing with Wash Buffer for 3 times (Wash Buffer were removed), 100 μL of TGF-β1 Conjugate were added to each well and incubated for 2h at RT. Following four washes, 100 μL of Substrate Solution were added and incubated for 30 min at RT. After adding Stop Solution (100 μL/well), the optical density was determined using plate reader set to 450nm. The exosomal TGF-β levels were determined using standard curves generated by plotting the Optical Density against free-ligand TGF-β standards. Data shown were means of triplicates ± SD and were representative of three separate experiments.

Expression of sTβRII-Fc by Ad-CMV-sTβRII-Fc virus

MDA-MB-231 cells were infected with control virus (Ad-CMV-Tomato virus at MOI: 1000) or Ad-CMV-sTβRII-Fc virus (MOI: 2000). After 48h, culture media replaced by serum free medium and maintained for further 48h. The culture media were harvested and heated at 95 °C for 10 minutes in reducing (R) or non-reducing (NR) buffer and were analysed for sTβRII-Fc expression by Western blotting. The sTβRII-Fc was detected by both anti-hIgG1-Fc antibody and anti-ecTβRII antibody. The yield of sTβRII-Fc was quantified by Coomassie Blue Staining. The final protein yield was determined as 2.5μg/mL using standard curve generated by plotting the protein mass and intensity of the BSA standards.

TGF-β signalling Inhibition Assay

Reporter cells (21D1) were seeded (3000 cells/well in 96-well plate) and co-infected with Ad-pCAGA-luciferase virus (MOI: 5000) and Ad-CMV-Gaussia-luciferase virus (MOI: 1000) for 24h. Thereafter, reporter cells were stimulated with indicated samples (exosomes or free-ligand TGF-β) in the presense of sTβRII-Fc or SB431542 for a further 24h. Cells were lysed and luciferase activities were assessed as described above. For inhibition by Smad7, reporter cells (21D1 cells) were seeded and co-infected with Ad-pCAGA-luciferase virus (MOI: 5000), Ad-CMV-Gaussia-luciferase virus (MOI: 1000) and Ad-CMV-Smad7 virus (MOI: 500 and 1000) or control virus (Ad-CMV-Tomato virus at MOI: 1000) for 24h before stimulated with 1ng/mL free-ligand TGF-β or 10μg/mL exosomes for further 24h. Cells were lysed and luciferase activities were assessed as described above. Data were presented as the fold change as compared with the basal reporter level or percentage of inhibition by sTβRII-Fc, SB431542 or Smad7. Data shown were means of triplicates ± SD and were representative of three separate experiments.

Cell co-culture

NIH3T3 fibroblasts were pre-seeded in 24-well plate (5×10^4 cells/well) while reporter cells (R1B, DR26 or T84) were pre-infected with Ad-pCAGA-luciferase virus (MOI: 5000) for 24h. Thereafter, reporter cells were washed three times with 1× PBS before re-seeded over the NIH3T3 fibroblasts (1×10^4 reporter cells/well). Cells in co-culture were treated with or without 5ng/mL free-ligand TGF-β. After 24h, cells were lysed and luciferase activities were assessed as described above. Data were presented as the fold change as compared with the

basal reporter level. Data shown were means of triplicates ± SD and were representative of three separate experiments.

Statistics

All statistical analyses were calculated by Student's t -test. Error bars indicate s.d (standard deviation).

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Figure Legends

Figure 1. TGF-β-positive exosomes secreted by MDA-MB-231 cells induce TGF-β-Smad signalling. **(A)** Differential centrifugation protocol for isolating exosomes from culture media of $1x10^8$ MDA-MB-231 (MDA) cells. Retentate I was washed twice with 1×PBS and concentrated using 100K NMWL centrifugal units to remove free-ligand TGF-β. **(B)** P100 fraction (exosomes) was subjected to cryo-TEM and Western blotting analysis (10μg) for exosome characterization. Scale bar, 100nm. **(C)** Reporter cells (NIH3T3 fibroblasts) were stimulated with 2ng/mL free-ligand TGF-β or 10μg/mL MDA exosomes. After 24h, luciferase activities were measured and data were presented as the fold change as compared with the basal reporter level. Relative luciferase activity represents TGF-β reporter activity. **(D)** NIH3T3 fibroblasts (2.5×10^5) were incubated with indicated dose of free-ligand TGF-β or MDA exosomes (10μg/mL) for 1h. Cells were lysed and the cell lysates were analysed for phospho-Smad2, total Smad2/3 and β-Actin expression by Western blotting. pSmad2 represents phospho-Smad2. tSmad2/3 represents total Smad2/3. **(E)** NIH3T3 cells (6×10^3) were incubated with 2ng/mL free-ligand TGF-β or MDA exosomes (10μg/mL) for 1h. Cells were fixed and processed for immunofluorescence staining with antibodies against Smad2 (mouse, 1:300, green). Nuclei were stained with Hochest (blue). Images were collected by Olympus FV1000 confocal microscope and processed using the FV1000 software (magnification = 40×). **(F)** 1.3×10^7 MDA cells were transfected with control siRNA or siRNA targeting Rab27a (13 nmol/L siRNA) for 48h and cultured in serum-free medium for further 24h for exosome production. Cells were lysed and analysed for Rab27a and β-Actin expression by Western blotting. Exosomes (exosomes enriched fraction: supernatant III in Fig. 1A) from these cells were prepared the same way as above and the levels of exosomal TGF-β were determined by TGF-β reporter activity assay. Molecular mass markers (in kilo Daltons) were shown to the left. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: **P <0.01, *** P <0.001.

Figure 2. Highly metastatic tumor cells exhibit higher levels of exosomal TGF-β. **(A, B)** Exosomes were harvested from culture media of indicated human cell lines using differential centrifugation protocol as outlined in Fig. 1A. The TGF-β reporter activities were measured in 21D1 reporter cells using the same way as in Fig. 1C and the exosomal TGF-β level was determined using standard curve generated by plotting the various concentrations of free-ligand TGF-β against the measured TGF-β reporter activities. The amounts of exosomes were quantified by BCA assay. Exosomal TGF-β levels were presented as per million cells (A) or per μg exosomes (B). **(C-E)** Exosomes were harvested from the culture media of 1×10^8 MDA-MB-231 or 3×10^8 MCF-7 cells treated with or without 5ng/mL free-ligand TGF-β for 24h followed by the same differential centrifugation protocol as outlined in Fig. 1A. Of note, Retentate I was washed three times with 1 × PBS and concentrated using 100K NMWL centrifugal units to remove free-ligand TGF-β. The exosomal TGF-β activities and amounts of exosomes were determined and presented. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: **P <0.01, *** P <0.001.

Figure 3. Exosomes transduce TGF-β signalling in TGF-β receptor-defective cells. **(A)** Reporter cells (R1B, DR26 and T84) were treated with or without indicated free-ligand TGF-β or MDA exosomes (50μg/mL) for 24 h. Cells were lysed and luciferase activities were assessed as described above. **(B)** 2×10^7HEK 293T cells were transfected with either TβRI or TβRII or both expression constructs for 48h and cultured in serum-free medium for further 24h for exosome production. Cells and exosomes (exosomes enriched fraction: supernatant III in Fig. 1A) were lysed and analysed for TβRI, TβRII and Alix expression by Western blotting. TβRI and TβRII were indicated by white and black arrowheads, respectively (black arrows, non-specific band). **(C-E)** NIH3T3 fibroblasts were pre-seeded while reporter cells (R1B, DR26 and T84) were pre-infected with Ad-pCAGA-luciferase virus (MOI: 5000) for 24 h. The reporter cells were washed three times with 1× PBS before re-seeded over the NIH3T3 fibroblasts. Cells in co-culture were treated with or without 5ng/mL TGF-β for 24h before luciferase assay were conducted. Data were presented as the fold change as compared with the basal reporter level. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001.

Figure 4. TGF-β ligand sequesters are not effective in blocking exosomal TGF-β. **(A)** MDA-MB-231 cells were infected with control virus (Ad-CMV-Tomato virus at MOI: 1000) or Ad-CMV-sTβRII-Fc virus (MOI: 2000). After 48h, culture media were replaced by serum free medium and maintained for further 48h. The culture media were heated at 95 °C for 10 minutes in reducing (R) or non-reducing (NR) buffer and were analysed for sTβRII-Fc expression by Western blotting. The sTβRII-Fc was detected by both anti-hIgG1-Fc antibody and anti-ecTβRII antibody. **(B)** Reporter cells (21D1 cells) were treated with indicated dose of free-ligand TGF-β or MDA exosomes in the presence of sTβRII-Fc (2.5 μg/mL) for 24h. Luciferase activities were measured and data were presented as the fold changes relative to the basal reporter level. **(C)** Data in (B) were presented as percentage of inhibition by sTβRII-Fc. **(D)** The levels of free-ligand TGF-β (0.5ng/mL) and exosomal TGF-β (exosomes: 3μg/mL) were measured by the TGF-β reporter activity and TGF-β1 ELISA assays. **(E, F)** Similar to (B, C) with SB431542 replacing sTβRII-Fc. **(G-H)** Reporter cells (21D1 cells) were seeded and infected Ad-CMV-Smad7 virus (MOI: 500 and 1000) or control virus (Ad-CMV-Tomato virus at MOI: 1000) for 24h before stimulated with 1ng/mL free-ligand TGF-β or 10μg/mL MDA exosomes for further 24h. Cells were lysed and luciferase activities were assessed as described above. Data were representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: ** P <0.01, *** P <0.001, **** P <0.0001.

Figure S1. Active TGF-β is present on exosomes secreted by MDA-MB-231 cells. **(A)** Exosomes were isolated from culture media of $1x10^8$ MDA-MB-231 (MDA) cells using differential centrifugation protocol as outlined in Fig. 1A. Reporter cells (21D1, NIH3T3 or MDA cells) were stimulated with same volume (10μL) of 10,000 × g supernatant or 100,000 × g supernatant for 24h. 10,000 × g supernatant was the supernatant after 10,000 × g centrifugation (supernatant III in Fig. 1A). 100,000 × g supernatant was the supernatant after 100,000 × g centrifugation (supernatant IV in Fig. 1A). After 24h, luciferase activities were measured and data were presented as the fold change as compared with the basal reporter level. Relative luciferase activity represents TGF-β reporter activity. **(B)** Reporter cells (21D1 and MDA cells) were stimulated with 2ng/mL free-ligand TGF-β or 10μg/mL MDA exosomes for 24h. Cells were lysed and luciferase activities were assessed as described above. **(C)** MDA cells (2.5×10^5) were incubated with 2ng/mL free-ligand TGF-β or 10μg/mL MDA exosomes for 1h. Cells were lysed and the cell lysates were analysed for phospho-Smad2, total Smad2/3 and β-Actin expression by Western blotting. pSmad2 represents phospho-Smad2. tSmad2/3 represents total Smad2/3. **(D)** MDA cells (6×10^3) were incubated with 2ng/mL free-ligand TGF-β or MDA exosomes (10μg/mL) for 1h. Cells were fixed and processed for immunofluorescence staining with antibodies against Smad2 (mouse, 1:300, green). Nuclei were stained with Hochest (blue). Images were collected by Olympus FV1000 confocal microscope and processed using the FV1000 software (magnification = 40×). **(E)** 1.3×10^7 MDA cells were transfected with control siRNA or siRNA targeting Rab27a (13 nmol/L siRNA) for 48h and cultured in serum-free medium for further 24h for exosome production. The indicated MDA exosomes (exosomes enriched fraction: supernatant III in Fig. 1A) were lysed and analysed by Western blotting for the expression of exosome marker TSG101 and Alix. Molecular mass markers (in kilo Daltons) were shown to the left. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: *P <0.05, **P <0.01, *** P <0.001.

Figure S2. Higher levels of exosomal TGF-β are observed in highly metastatic tumor cells. **(A)** Exosomes were harvested from culture media of indicated human cell lines using differential centrifugation protocol as outlined in Fig. 1A. The amounts of exosomes were quantified by BCA assay. Exosome amounts (μg) were presented as per million cells. **(B-D)** Exosomes were harvested from culture media of indicated human cell lines using differential centrifugation protocol shown in Fig. 1A. The levels of exosomal TGF-β were determined by TGF-β reporter activity assay. Data were presented as the fold change as compared with the basal reporter level. **(E)** Exosomes were harvested from culture media of 1×10^8 MDA-MB-231 or 3×10^8 MCF-7 cells treated with or without 5ng/mL free-ligand TGF-β by the same way as in Fig. 2C-2E. Reporter cells (21D1 cells) were stimulated with indicated fractions for 24h. Luciferase activities were measured and data were presented as the fold change as compared with the basal reporter level. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: **P <0.01, *** P <0.001.

Figure S3. (A) NIH3T3 fibroblasts secrete TGF-β-positive exosomes. Exosomes were harvested from culture media of NIH3T3 fibroblasts (1.5×10^8) using differential centrifugation protocol as outlined in Fig. 1A. The TGF-β reporter activities were measured in 21D1 reporter cells and exosomal TGF-β levels were determined using standard curve generated by plotting the various concentrations of free-ligand TGF-β against the measured TGF-β reporter activities. The amounts of exosomes were quantified by BCA assay. Exosomal TGF-β levels were presented as per million cells or per μg exosomes. **(B)** Quantification of sTβRII-Fc by Coomassie Blue Staining. MDA-MB-231 cells were infected with control virus (Ad-CMV-Tomato virus at MOI: 1000) or Ad-CMV-sTβRII-Fc virus (MOI: 2000). After 48h, culture media were replaced by serum free medium and maintained for further 48h. The culture media were heated at 95 °C for 10 minutes in non-reducing (NR) buffer and the yield of sTβRII-Fc was quantified by Coomassie Blue Staining. The sTβRII-Fc band was indicated by black arrow. The final protein yield was determined as 2.5 μg/mL using standard curve generated by plotting the protein mass and intensity of the BSA standards. **(C)** Standard curves for TGF-β reporter activity assay and TGF-β1 ELISA assay. A range of free-ligand TGF-β concentrations were measured by TGF-β reporter activity assay and TGF-β1 ELISA assay. Standard curves were generated by plotting the Relative Light Unit or Optical Density against free-ligand TGF-β concentrations. The exosomal TGF-β levels were determined using standard curves. Insets are magnified images of the boxed

region. **(D)** Expression of Smad7 by using Ad-CMV-Smad7 virus. 21D1 cells were infected with Ad-CMV-Smad7 virus (MOI: 500 and 1000). After 24h, cells were lysed and the cell lysates were analysed for Smad7 (FLAG-Smad7, detected by anti-FLAG (M2) antibody) and β-Actin expression by Western blotting. Molecular mass markers (in kilo Daltons) were shown to the left. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates.

Figures

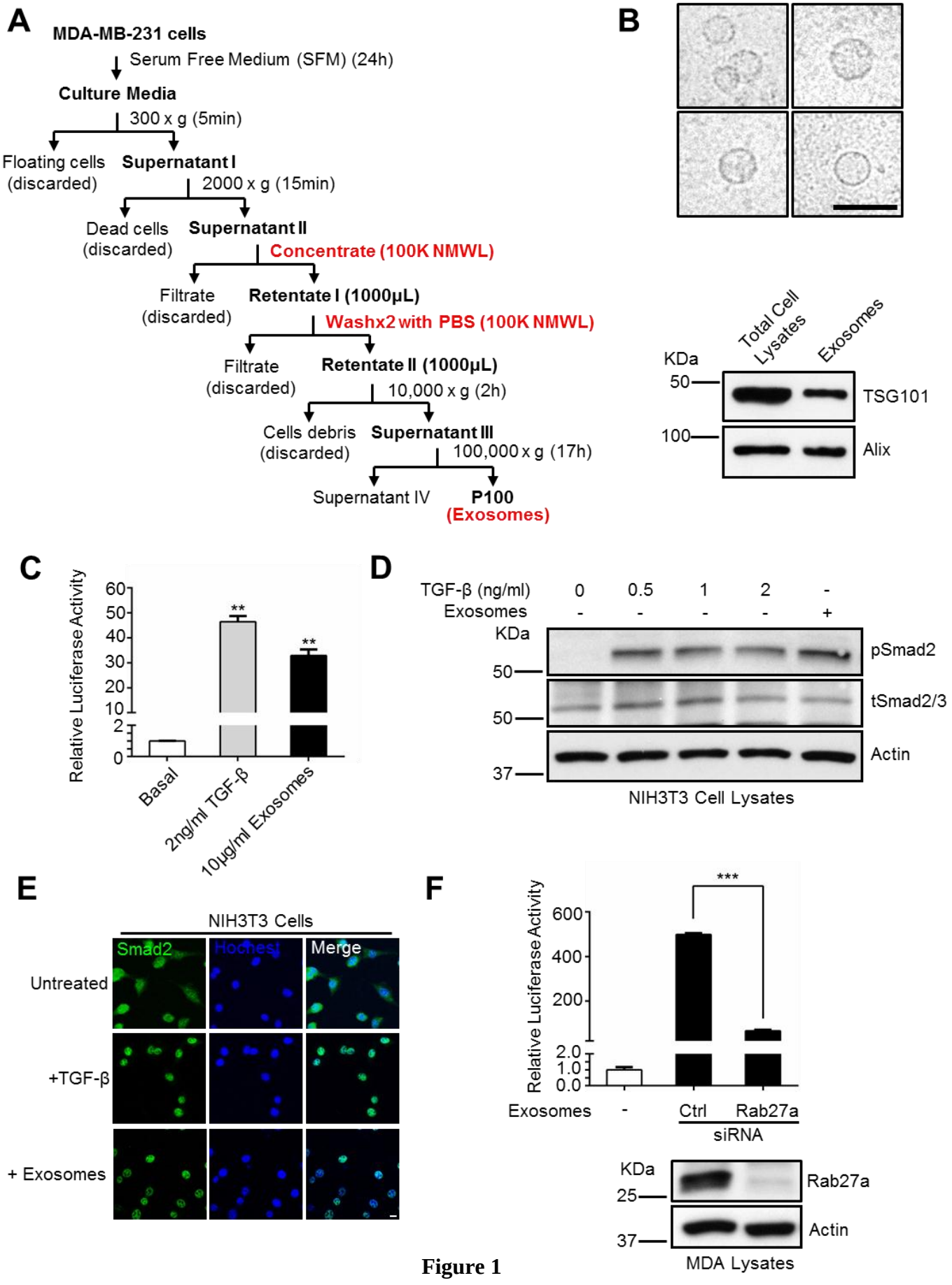

Figure 1

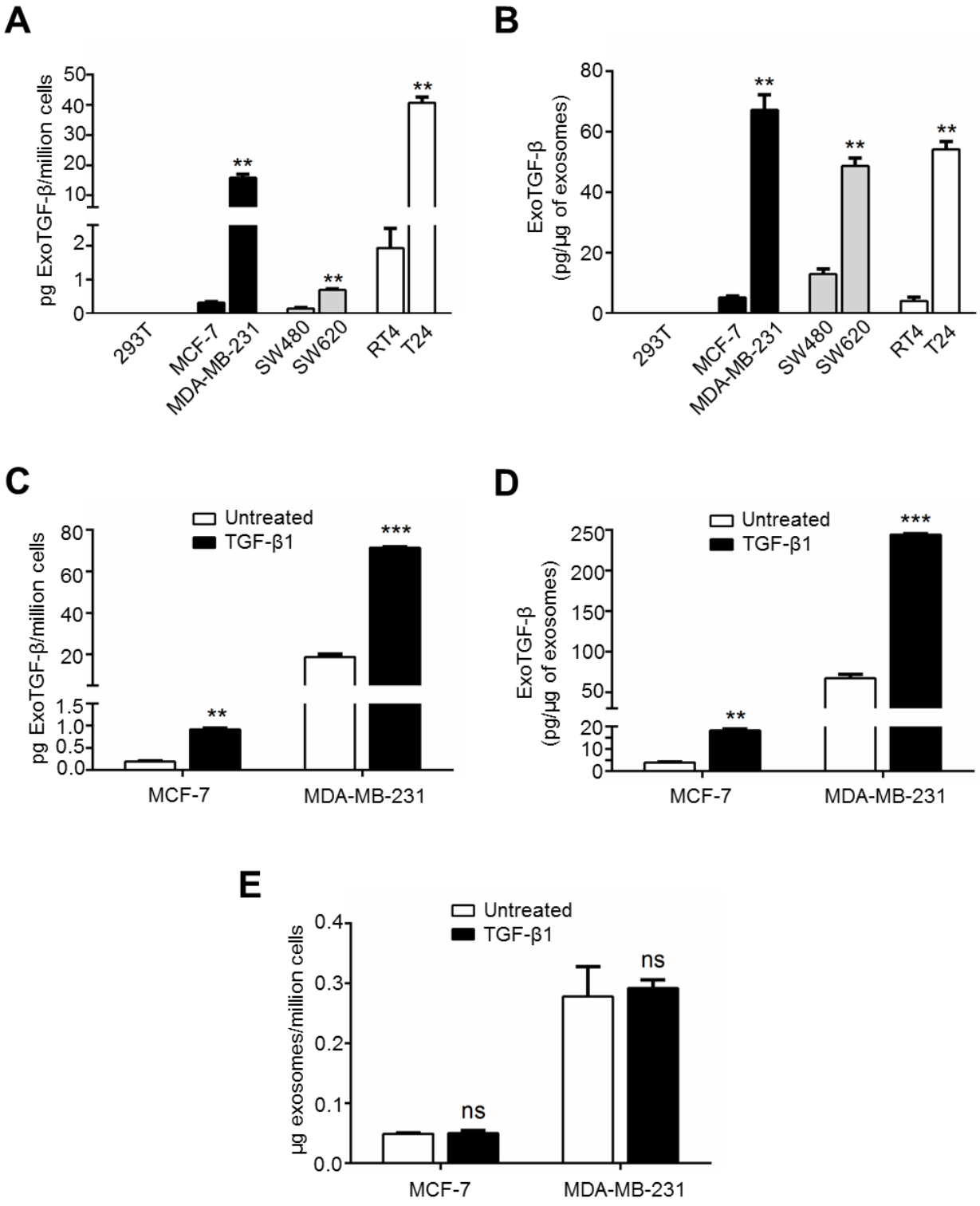
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Figure 2

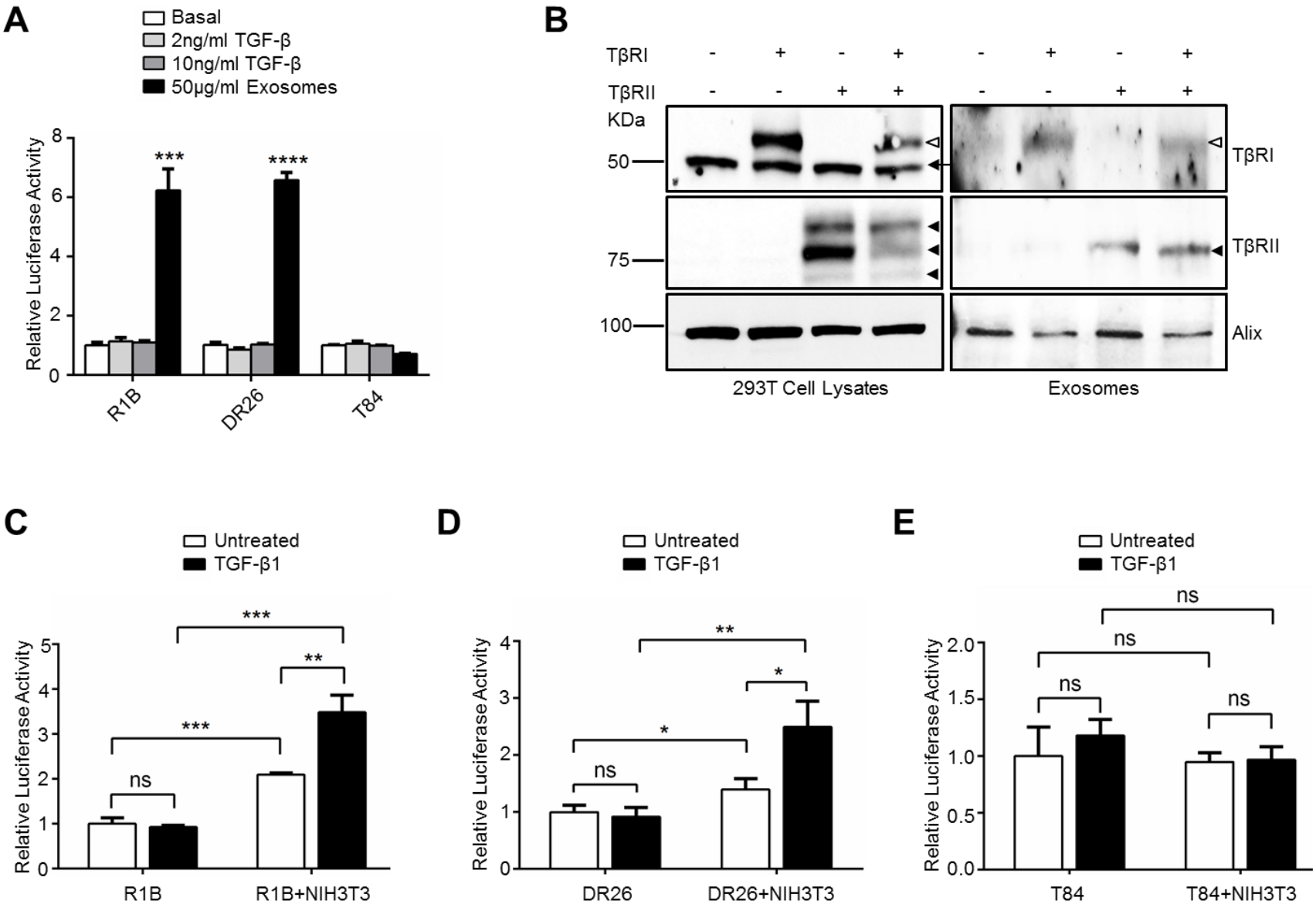
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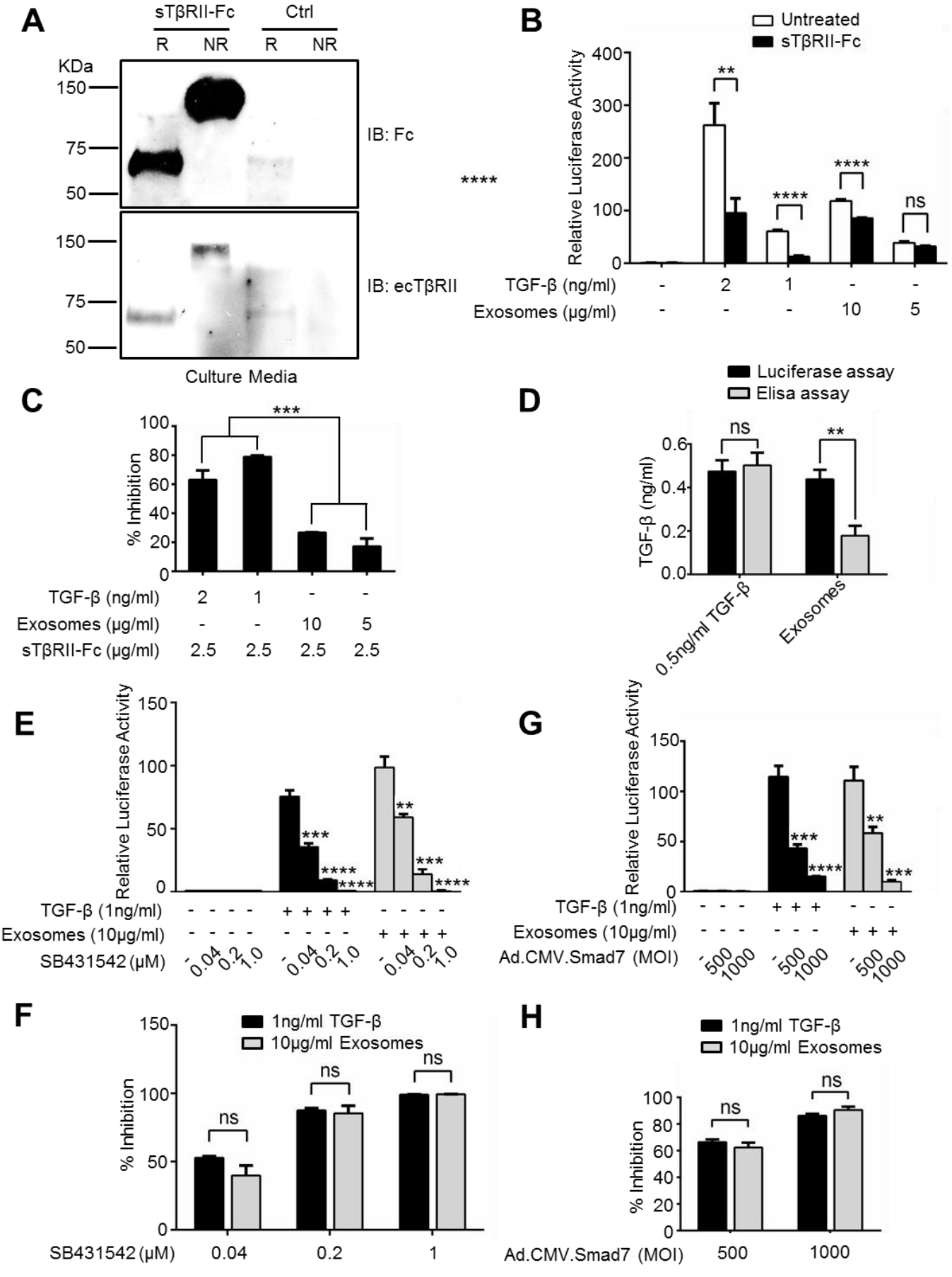
Figure 3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TGF-β (ng/ml) - 2 1 - -
Exosomes (μg/ml) - - - 10 5
C
***
% Inhibition
100
80
60
40
20
0
TGF-β (ng/ml) 2 1 - -
Exosomes (μg/ml) - - 10 5
sTβRII-Fc (μg/ml) 2.5 2.5 2.5 2.5
D
Luciferase assay
Elisa assay
TGF-β (ng/ml)
0.6
0.4
0.2
0.0
ns
**
0.5ng/ml TGF-β
Exosomes
E
Relative Luciferase Activity
150
100
50
0
TGF-β (1ng/ml) - - - - + + + + - - - -
Exosomes (10μg/ml) - - - - - - - - + + + +
SB431542 (μM) 0.04 0.2 1.0 0.04 0.2 1.0 0.04 0.2 1.0
G
Relative Luciferase Activity
150
100
50
0
TGF-β (1ng/ml) - - - + + + - - -
Exosomes (10μg/ml) - - - - - - + + +
Ad.CMV.Smad7 (MOI) 500 1000 500 1000 500 1000
F
1ng/ml TGF-β
10μg/ml Exosomes
% Inhibition
150
100
50
0
ns
ns
ns
SB431542 (μM) 0.04 0.2 1
H
1ng/ml TGF-β
10μg/ml Exosomes
% Inhibition
100
80
60
40
20
0
ns
ns
Ad.CMV.Smad7 (MOI) 500 1000

Figure 4

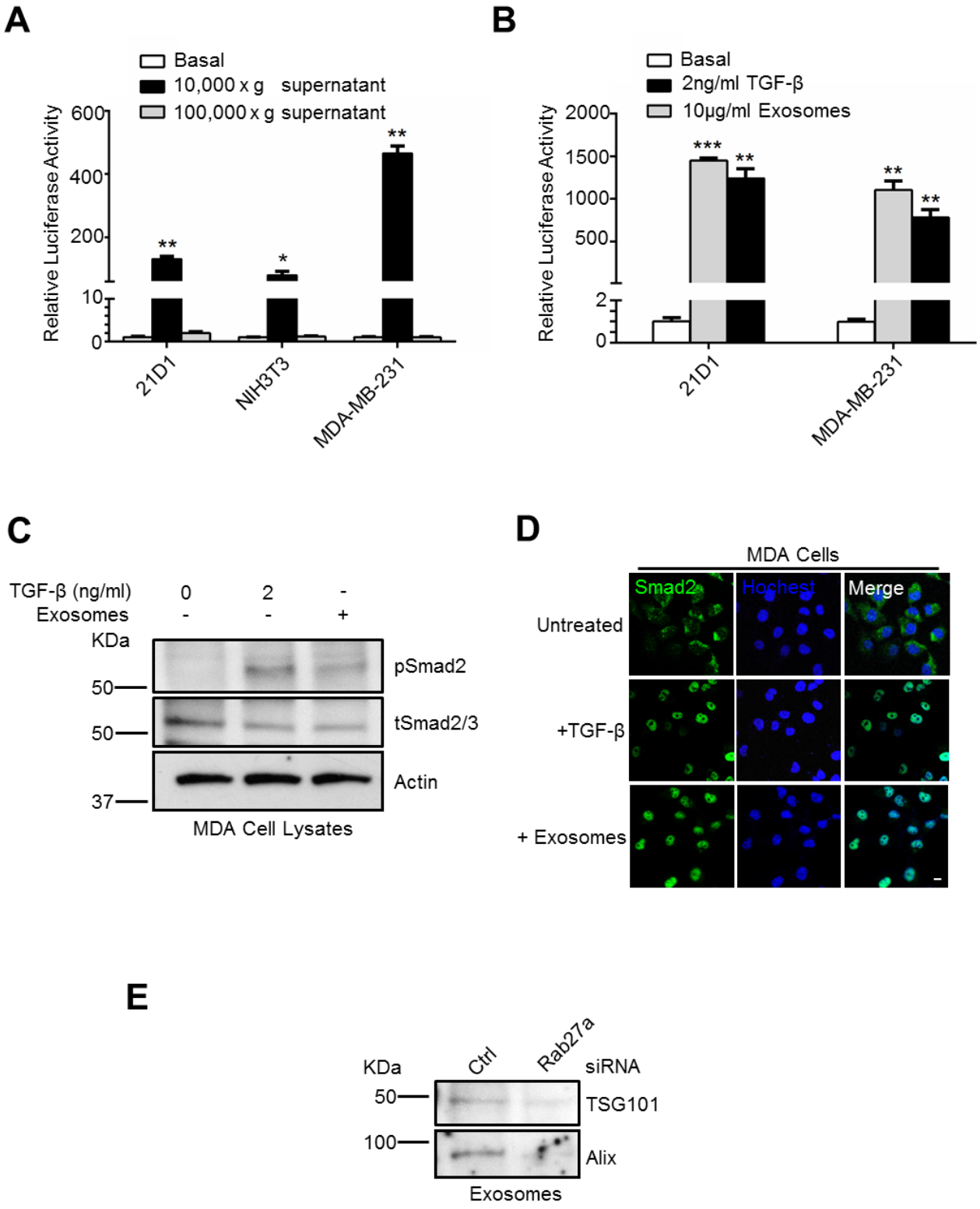
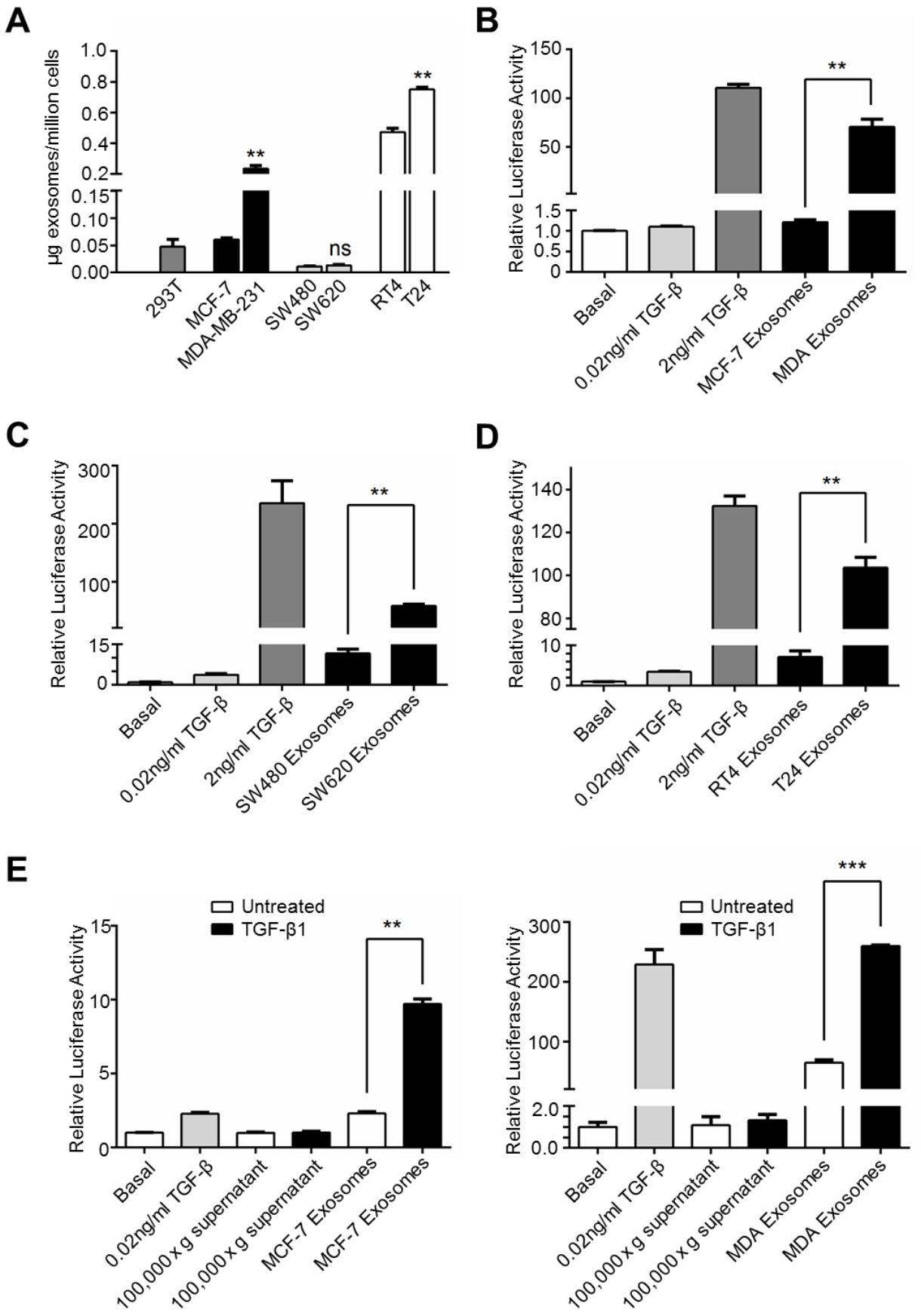

A
Basal
10,000 x g supernatant
100,000 x g supernatant
Relative Luciferase Activity
600
400
200
10
0
**
*
**
21D1
NIH3T3
MDA-MB-231
B
Basal
2ng/ml TGF-β
10μg/ml Exosomes
Relative Luciferase Activity
2000
1500
1000
500
2
0
***
**
**
**
21D1
MDA-MB-231
C
TGF-β (ng/ml) 0 2 -
Exosomes - - +
KDa
50
50
37
pSmad2
tSmad2/3
Actin
MDA Cell Lysates
D
MDA Cells
Smad2
Hochest
Merge
Untreated
+TGF-β
+ Exosomes
E
KDa
Ctrl
Rab27a
siRNA
50
100
TSG101
Alix
Exosomes

Figure S1


A
μg exosomes/million cells
1.0
0.8
0.6
0.4
0.2
0.15
0.10
0.05
0.00
**
ns
**
293T
MCF-7
MDA-MB-231
SW480
SW620
RT4
T24
B
Relative Luciferase Activity
150
100
50
1.5
1.0
0.5
0
**
Basal
0.02ng/ml TGF-β
2ng/ml TGF-β
MCF-7 Exosomes
MDA Exosomes
C
Relative Luciferase Activity
300
200
100
15
0
**
Basal
0.02ng/ml TGF-β
2ng/ml TGF-β
SW480 Exosomes
SW620 Exosomes
D
Relative Luciferase Activity
140
120
100
80
10
0
**
Basal
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2ng/ml TGF-β
RT4 Exosomes
T24 Exosomes
E
Untreated
TGF-β1
Relative Luciferase Activity
15
10
5
0
**
Basal
0.02ng/ml TGF-β
100,000 x g supernatant
100,000 x g supernatant
MCF-7 Exosomes
MCF-7 Exosomes
Untreated
TGF-β1
Relative Luciferase Activity
300
200
100
2.0
0.0
***
Basal
0.02ng/ml TGF-β
100,000 x g supernatant
100,000 x g supernatant
MDA Exosomes
MDA Exosomes

Figure S2

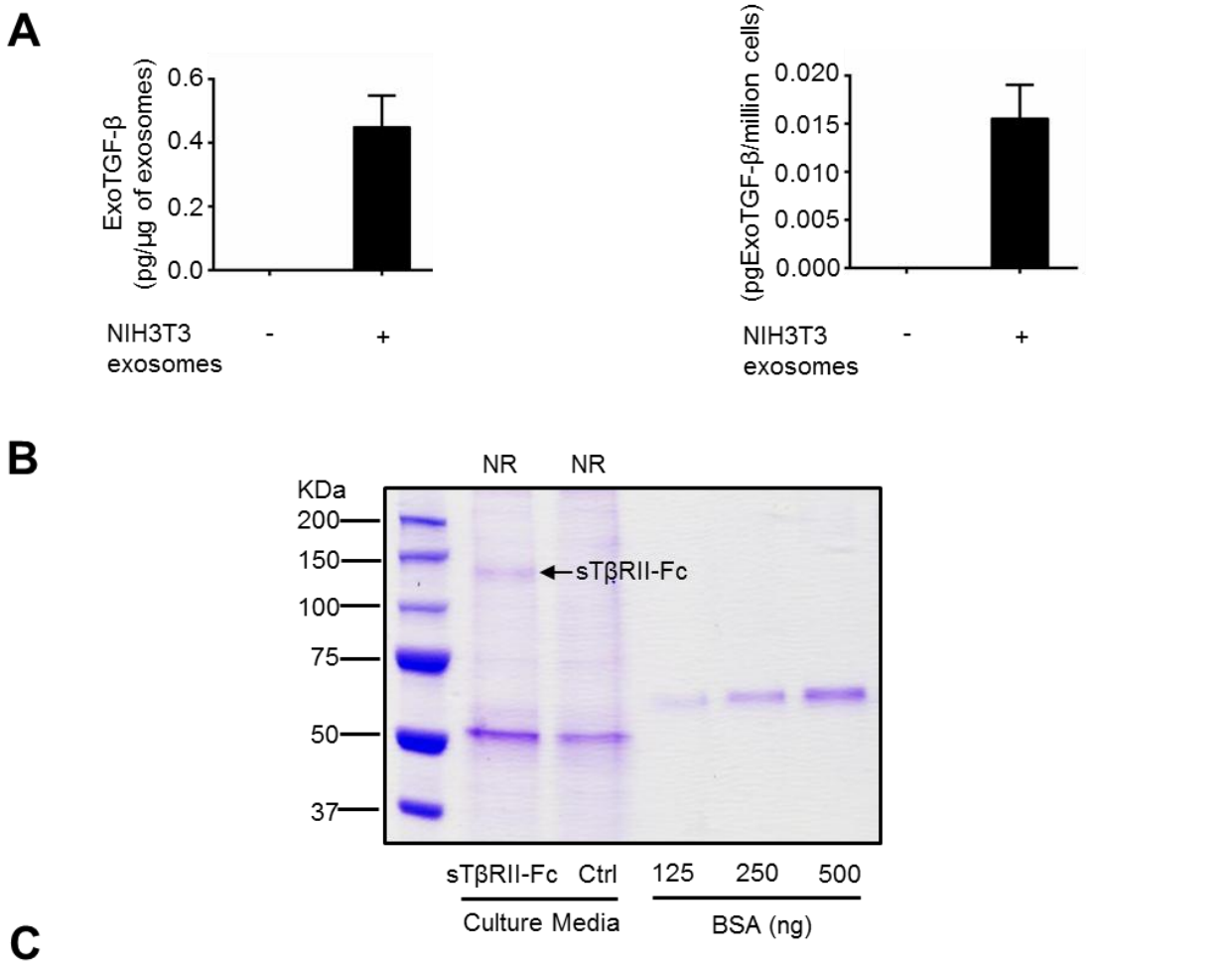
A
ExoTGF-β (pg/μg of exosomes)
0.6
0.4
0.2
0.0
NIH3T3 exosomes - +
(pgExoTGF-β/million cells)
0.020
0.015
0.010
0.005
0.000
NIH3T3 exosomes - +
B
NR NR
KDa
200
150
100
75
50
37
sTβRII-Fc
sTβRII-Fc Ctrl
Culture Media
125 250 500
BSA (ng)
C
Relative Light Unit (x10⁶)
1.5
1.0
0.5
0.0 0.5 1.0 1.5 2.0 2.5
TGF-β (ng/ml)
(x10⁴)
2.50
2.00
1.50
1.00
0.50
0.00 0.02 0.04 0.06 0.08
Optical Density
1.5
1.0
0.5
0.0 0.5 1.0 1.5 2.0 2.5
TGF-β (ng/ml)
0.3
0.2
0.1
0.0 0.1 0.2 0.3
D
Ad.CMV.Smad7(MOI) 0 500 1000
KDa
50
37
Smad7
Actin

Figure S3

Chapter 4.

Ras-dependent exosomal discharge of cytosolic protein SPSB1

Ras-dependent exosomal discharge of cytosolic protein SPSB1

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Abstract

Exosomes are nanometre-sized vesicles that contain a broad range of bioactive molecules. Exosomes participate in intercellular communication and modulate the behaviour of recipient cells, but their biological effects on donor cells are poorly understood. It has been shown that Ras enhances transforming growth factor-β (TGF-β) signalling by down-regulating the TGF-β type II receptor (TβRII) negative regulator, the cytosolic protein SPSB1. However, the molecular and cellular mechanism by which Ras may decrease the intracellular SPSB1 levels is yet to be determined. Here, we report that exosomes are involved in Ras-induced down-regulation of SPSB1 by exporting SPSB1 from the cell. Ras-mediated SPSB1's MVB sorting and exosome secretion are dependent on ceramide, PI3K and intracellular Ca^{2+} concentration. SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain is necessary for SPSB1's recruitment to CD63-positive late endosome and exosomes, while the ubiquitination of SOCS box is not required. Moreover, the SPSB1- and Ras-containing exosomes can be internalized by recipient cells. Taken together, our results demonstrate that exosomal discharge modulates the abundance of cytosolic contents, and also imply the involvement of exosomes in Ras-mediated up-regulation of TGF-β signalling.

Introduction

Exosomes are small membranous vesicles (40-100nm in diameter) secreted by various cell types [1]. They are generated from late endosomes/multivesicular bodies (MVBs) [2] and are released into the extracellular space upon the fusion of MVBs outer membrane with the plasma membrane [3]. The endosomal sorting complex required for transport (ESCRT) machinery, ceramide, PI3K as well as intracellular Ca^{2+} concentration have been implicated in endosomal-exosomal sorting. Exosomes carry a broad range of bioactive cargoes (such as proteins, genetic materials, and lipids) and exert biological effects on recipient cells. As such, exosomes are involved in the progression of human disease, including cancer. However, how exosomes may affect the behaviour of donor cells is poorly understood.

Studies have revealed the role of exosomes in mediating the discharge of specific cytosolic proteins, providing a new insight into the control of intracellular protein levels in addition to the canonical protein degradation mechanism (via proteasome and lysosome). Importantly, exosome-mediated protein clearance has been found to alter the signalling activity of donor cells. For example, β-catenin is secreted in association with exosomes upon tetraspannins CD9 and CD82 overexpression, causing the down-regulation of Wnt-β-catenin signalling [2]. The tetraspannin CD63 is found to antagonize the constitutive NF-κB activation via enhancing the exosomal export of LMP1 from the cells [4]. Moreover, exosomal packaging and release of tumor suppressor-miRNAs have been observed in highly metastasis bladder cancer cell lines, which enhance the metastatic potential of tumor cells [5].

Transforming growth factor-β (TGF-β) is a key regulator in cancer progression. In normal or premalignant cells, TGF-β signalling exerts the tumor-suppressive effects. However, malignant tumors circumvent the tumor-suppressive functions of TGF-β signalling while exploit TGF-β signalling to promote tumor aggressiveness [6]. TGF-β signalling is tightly regulated by multiple molecules at each step of the signalling cascade. In particular, the cytosolic protein SPRY domain and SOCS Box containing protein 1 (SPSB1) has been identified as a negative regulator of TGF-β signalling by targeting TGF-β type II receptor (TβRII) for ubiquitin-dependent degradation [7]. The SPRY domain of SPSB1 is recognized as a protein interaction module which regulates innate and adaptive immunity [8]. The SOCS box binds to elongin B and elongin C, cullin-5 and RING-box-2 (RBX2), resulting in the recruitment of E2 ubiquitin transferase [9].

It has been shown that Ras signalling cooperates with TGF-β signalling in tumorigenesis. For example, Ras-induced activation of MAPK/ERK signalling down-regulates TβRII in lung cancer by recruiting transcription repressors to TβRII promoter [10]. On the other hand, Ras enhances TGF-β signalling via targeting TβRII negative regulator, the cytosolic protein SPSB1 (Liu et al., manuscript in reviewing). SPSB1 interacts with Ras through its SPRY domain, which is abolished by the mutation at residue Y129. Ras expression triggers a reduction in the intracellular levels of SPSB1, which in turn stabilizes TβRIIs and results in elevated TGF-β signalling activity. However, the molecular and cellular mechanism by which Ras may decrease the SPSB1 levels is controversial as Ras does not change the poly-ubiquitination of SPSB1.

Considering the role of exosomes in intracellular protein clearance, together with the observation that SPSB1 is readily detected in exosomes from Ras-transformed cells (unpublished data), we hypothesized that exosomes may be involved in Ras-induced down-regulation of SPSB1 by exporting SPSB1 from the cells. We found that SPSB1 was secreted in association with exosomes upon Ras overexpression, resulting in a reduction in the cellular pool of SPSB1. Treatments using small molecular inhibitors indicated that Ras-mediated SPSB1's MVB sorting and exosome secretion were dependent on ceramide, PI3K and intracellular Ca^{2+} concentration. SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain was necessary for SPSB1's recruitment to CD63-positive late endosome and exosomes, while the ubiquitination of SOCS box was not required. Additionally, the SPSB1- and Ras- containing exosomes could be internalized by recipient cells. Collectively, this study highlights the role of exosomes as vehicles for intracellular protein clearance, and also suggests the involvement of exosomes in Ras-mediated up-regulation of TGF-β signalling.

Results

Ras reduces the intracellular/cytosolic levels of SPSB1 by mediating their export via exosomes.

To test the possibility that Ras reduces the intracellular/cytosolic levels of SPSB1 by facilitating their transport into exosomes, we analysed the exosomal fractions from HEK 293T cells transiently transfected with plasmid encoding FLAG-SPSB1 alone or in combination with plasmid encoding Ras for the presence of SPSB1. Exosomes were purified using a differential centrifugation protocol [11] (Fig. 1A) and were characterized. Cryo-transmission electron microscopy (cryo-TEM) analysis suggested that P100 fraction exhibited exosome-typical size (40-100nm) and morphology. Immunoblotting revealed the presence of SPSB1, Ras and exosome markers (TSG101, Alix and CD81) in P100 fraction (exosomes) of HEK 293T cells expressing SPSB1 and Ras (Fig. 1B).

As shown in Fig. 1C, Ras overexpression resulted in a marked decrease in the intracellular levels of SPSB1, whereas SPSB1 were enriched in the exosomes. SPSB1 was detectable in exosomes only when Ras was co-expressed, indicating that the recruitment of SPSB1 into exosomes was Ras-dependent. Collectively, these data demonstrate that Ras reduces the cellular pool of SPSB1 by mediating SPSB1’s export via exosomes.

Ras-mediated SPSB1's endosomal-exosomal trafficking is dependent on intracellular Ca^{2+} concentration, ceramide and PI3K.

Multiple mechanisms have been implicated in intraendosomal membrane transport and exosome release. Previous studies have revealed that exosome secretion can be influenced by intracellular Ca^{2+} concentration. Thus, we tested the effect of Monensin, a membrane permeable Na^{+}/H^{+} exchanger which changes intracellular calcium concentration and stimulates exosome release [12, 13], on Ras-mediated exosomal export of SPSB1. Monensin appeared to enhance the Ras-induced exosome export of SPSB1 in a dose-dependent manner. Consistently, the intracellular SPSB1 levels were further decreased by Monensin treatment (Fig. 2A). Monensin was insufficient to reduce the cytosolic levels of SPSB1 when they were expressed alone, confirming our previous conclusion that Ras was required for the transport of SPSB1 into exosomes (Fig. 2B). A reduction of SPSB1 was observed in exosomes after treatment with DMA, an inhibitor of Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchanger which inhibits exosome secretion [14]. DMA treatment also caused a slight increase of intracellular SPSB1 levels (Fig. 2C). These data demonstrate the involvement of calcium-dependent mechanism in Ras-mediated exosome release of SPSB1.

The ceramide-dependent and PI3K-dependent exosome biogenesis can be blocked by the ceramidase inhibitor GW4869 and PI3K inhibitor wortmannin, respectively [15, 16]. After treatment with GW4869 and wortmannin, SPSB1 decreased in the exosomes, indicating the requirement of ceramide and PI3K for Ras-mediated exosomal secretion of SPSB1. Notably, neither GW4869 treatment nor wortmannin treatment significantly increased intracellular SPSB1 levels (Fig. 2D) and this finding was consistent in many repeated experiments, suggesting that other mechanism might also participate in SPSB1's exosome release.

Exosomal release of SPSB1 requires SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain.

As shown in Fig. 1C, the recruitment of SPSB1 into exosomes was Ras-dependent. SPSB1 interacts with Ras through its SPRY domain, which is abolished by the mutation at residue Y129 (Liu et al., manuscript in reviewing). To determine whether the interaction with Ras is important for its transport into exosomes, we compared the abundance of SPSB1 and the interaction defective SPRY domain mutant (SPSB1-129) in exosomes in the presence or absence of Ras. SPSB1 (MYC-SPSB1) was detectable in exosomes when Ras was co-expressed, which was consistent with our earlier observations (Fig. 3B, first row). However, SPSB1-129 (MYC-SPSB1-129) was barely detected in exosomes derived from HEK 293T cells co-transfected with plasmid encoding MYC-SPSB1-129 and plasmid encoding Ras. The intracellular levels of SPSB1-129 did not change upon Ras expression (Fig. 3B, second row). These results demonstrate that the exosome-associated release of SPSB1 is dependent on its interaction with Ras through SPRY domain.

The SOCS box of SPSB1 is characterized as functional domain which mediates the proteasomal degradation of TβRII. We next investigated whether the SOCS box of SPSB1 is also required for SPSB1's exosome release. To this end, HEK 293T cells were transfected with plasmid encoding the SOCS box deletion mutant (MYC-SPSB1Δ) alone or in combination with plasmid encoding Ras. The results showed that the deletion of SOCS box totally abolished the exosomal secretion of SPSB1 regardless of Ras expression, as demonstrated by the absence of SPSB1 in exosomes (Fig. 3B, third row). These data suggest the necessity of SOCS box for Ras-mediated exosome release of SPSB1.

SPSB1 accumulates in perinuclear vesicular structures in the presence of Ras.

We next investigated the subcellular localization of SPSB1 and the mutants (SPSB1-129, SPSB1Δ) in the presence or absence of Ras. As illustrated in Fig. 4A, when expressed alone, SPSB1 and SPSB-129 displayed a punctuate distribution throughout the cell, while SPSB1Δ diffusely distributed in the cytoplasm. Consistent with previous reports, Ras (v-Ha-Ras) was present at plasma membrane and endosomal compartments [17].

Interestingly, when Ras was co-expressed, SPSB1 accumulated in perinuclear vesicular structures, exhibiting a substantial degree of co-localization with Ras (Fig. 4B, first row). However, Ras overexpression had no impact on the subcellular localization of SPSB-129 and SPSB1Δ. A small proportion of SBSP1Δ was observed in Ras-positive regions, whereas SPSB-129 showed little co-localization with Ras (Fig. 4B, second row and third row). Collectively, these data demonstrate that Ras expression causes the accumulation of SPSB1 in perinuclear vesicular structures, which is dependent on SPSB1-Ras interaction and the SOCS box of SPSB1.

Ras promotes the recruitment of SPSB1 to CD63-positive late endosomes/MVBs.

As shown above, SPSB1 accumulated in perinuclear vesicular structures when Ras was co-expressed. Based on our previous observation that SPSB1 was detectable in exosomes in the presence of Ras (Fig. 1C), and because exosomes originate from late endosomes/multivesicular bodies (MVBs) [2], we speculated that these perinuclear vesicular structures were late endosomes/MVBs.

To test this possibility, we examined the co-localization of SBSB1 or SBSP1Δ with CD63, a late endosome/MVBs marker. As shown in Fig. 5, in the absence of Ras, little SPSB1 co-localized with CD63-positive late endosomes/MVBs. As expected, when Ras was co-expressed, SPSB1 was abundantly associated with the late endosomes. In contrast, SBSP1Δ had no co-localization with CD63-positive late endosomes/MVBs regardless of Ras expression. Thus, this result demonstrates that Ras promotes the recruitment of SPSB1 to CD63-positive late endosomes/MVBs before their secretion on exosomes. The accumulation of SPSB1 in the late endosome/MVBs depends on its interaction with Ras, and also the SOCS box.

The ubiquitination of SOCS box is not required for Ras-induced recruitment of SPSB1 into exosomes.

We next explored the molecular mechanism by which the SOCS box might mediate SPSB1's endosomal-exosomal sorting. It has been well documented that the ubiquitination of cargo serves as a signal for MVB sorting [18] and the subsequent exosome release. For example, the ubiquitination of PTEN is responsible for its transport into the exosomes [19]. The SOCS box of SPSB1 contains only one lysine, which, in principle, can serve as an acceptor site for ubiquitin. To test the possibility that the ubiquitination of SOCS box is responsible for the recruitment of SPSB1 into exosomes, we mutated Lys^{267} of SPSB1 (MYC-SPSB1) to arginine (MYC-SPSB1-267) to block the formation of ubiquitin chain (Fig. 6A) and examined its exosomal transport.

As shown in Fig. 6B, the replacement of lysine with arginine had no effect on the exosome release of SPSB1. In the presence of Ras, SPSB1-267 mutant was detectable in exosomes while showed a marked decrease in the cell lysates, similar to the results obtained for the wild-type SPSB1. In addition, SPSB1-267 mutant displayed a similar subcellular localization as the wild-type SPSB1 (Fig. 6C). SPSB1-267 exhibited a punctuate distribution throughout the cell when expressed alone. The accumulation of SPSB1-267 in Ras-positive perinuclear regions was apparent when Ras was co-expressed. Taken together, these results indicate that the SOCS box-mediated exosome secretion of SPSB1 is not due to the ubiquitination of SOCS box. Instead, the SOCS box may be able to recruit ubiquitin ligase complex and cause the ubiquitination of SPRY domain, which serves as the signal for SPSB1's sorting into MVBs and exosomes.

SPSB1-267 mutant co-localizes with CD63-positive late endosomes/MVBs in the presence of Ras.

To confirm that when Ras is co-expressed, SPSB1-267 mutant, similarly to the wild-type SPSB1, co-localizes with the CD63-positive late endosomes/ MVBs, we performed the confocal immunofluorescent analysis (Fig. 7). As expected, in the absence of Ras, the large majority of SPSB1 and SPSB1-267 didn't co-localize with CD63. However, the transient transfection of plasmid encoding Ras resulted in a significant accumulation of SPSB1 as well as SPSB1-267 in the CD63-positive late endosomes/ MVBs. In summary, SPSB1 and SPSB1-267 behave similarly when Ras is co-expressed, further confirming that the ubiquitination of SOCS box is not required for the recruitment of SPSB1 into exosomes.

Exosome-mediated transfer of SPSB1 and Ras into recipient cells.

Exosomes have been well characterized as crucial mediators of intercellular communication by transferring various contents between cells. Upon uptake, they exert their biological effects on the recipient cells. We next investigated whether SPSB1- and Ras- containing exosomes can be internalized by recipient cells.

Exosomes were isolated from untransfected HEK 293T cells or HEK 293T cells transiently co-transfected with plasmid encoding FLAG-SPSB1 and plasmid encoding Ras (v-Ha-Ras). These exosomes were then incubated with MDA-MB-231 cells in which endogenous SPSB1 and Ras (v-Ha-Ras) were undetectable. The uptake of SPSB1- and Ras- containing exosomes by MDA-MB-231 cells were confirmed by immunofluorescence staining. As shown in Fig. 8, SPSB1 and Ras were detected in the cytoplasm and plasma membrane of recipient MDA-MB-231 cells. However, the immunofluorescence of SPSB1 and Ras were absent when MDA-MB-231 cells were incubated with exosomes released by untransfected HEK 293T cells. These results demonstrate the exosomal transfer of SPSB1 and Ras into recipient cells.

Discussion

Exosomes have been increasingly recognized as crucial mediators in intercellular communication. Studies have been focused on exosome composition, biogenesis, their cargoes and their biological effects on the recipient cells, but how exosomes may affect the behaviour of donor cells is poorly understood. Here we show that exosomes are involved in the Ras-induced down-regulation of SPSB1 (TβRII negative regulator) by exporting SPSB1 from the cells (Fig. 9). We found that the cytosolic protein SPSB1 was secreted in association with exosomes upon Ras overexpression, triggering a reduction in the cellular pool of SPSB1. Ras-mediated SPSB1's MVB sorting and exosome secretion were dependent on ceramide, PI3K and intracellular Ca^{2+} concentration. SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain controlled SPSB1's co-localization with CD63-positive late endosome and exosome release, while the ubiquitination of SOCS box was not required. Moreover, the SPSB1- and Ras- containing exosomes could be internalized by recipient cells. Collectively, our work highlights the role of exosomes as vehicles for intracellular protein clearance, and also suggests the involvement of exosomes in Ras-mediated up-regulation of TGF-β signalling.

In general, the intracellular protein levels are maintained tightly through the balance between protein synthesis and degradation. Exosome discharge of cytosolic proteins provides a new mechanism in addition to canonical protein degradation mechanism (via proteasome and lysosome). Examples of the exosome-mediated protein clearance and alteration in the signalling activity of donor cells have been documented previously. The β-catenin is secreted in association with exosomes upon tetraspannins CD9 and CD82 overexpression, causing the down-regulation of Wnt-β-catenin signalling [2]. The tetraspannin CD63 is found to antagonize the constitutive NF-κB activation via enhancing the exosomal export of LMP1 from the cells [4]. Our observation adds to the accumulation of evidences for exosome-mediated intracellular protein clearance. Ras mediates the loading SPSB1, a TβRII negative regulator, into exosomes for secretion, therefore reducing its cellular levels and enhancing TGF-β signalling. It has been shown that Ras recruits PI3K to endosome and induces their signalling activity [20]. Our data showed that treatment of cells with PI3K inhibitor wortmannin markedly reduced the exosome release of SPSB1, indicating the involvement of PI3K in this process. Notably, although a decreased exosome secretion of SPSB1 was observed after wortmannin and GW4869 (ceramide inhibitor) treatments, the intracellular

SPSB1 levels were not significantly affected (Fig. 2D), suggesting that other mechanism may be in operation.

Our results demonstrated that SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain was required for SPSB1's MVB sorting and exosome secretion. The single mutation (Y129A) in the SPRY domain of SPSB1 blocked not only its interaction with Ras (Liu et al., manuscript in reviewing) but also the exosome sorting. In addition, the SOCS box deletion mutant was observed to disrupt Ras-mediated transport of SPSB1 into exosomes (Fig. 3-Fig. 5). As such, either the interaction with Ras or SOCS box alone is not sufficient for the secretion of SPSB1 by exosomes, needing both elements. SPSB1 is likely not the only molecule involved in the exosome-mediated cellular protein discharge mechanism which is dependent on Ras. Other possible candidates may include the SPRY domain and SOCS box-containing protein family members (such as SPSB2-4), and also the SOCS box-containing proteins which interact with Ras. How dose SOCS box mediate SPSB1's exosome sorting? It has been well established that the ubiquitination of cargo can serve as a signal in MVB sorting [18] and the subsequent exosome secretion. For example, the ubiquitination of PTEN is responsible for its transport into exosomes [19]. The SOCS box of SPSB1 contains only one lysine, which, in principle, can serve as an acceptor site for ubiquitin. However, replacement of this lysine with arginine (K267R) didn't affect SPSB1's exosome release (Fig. 6), indicating that ubiquitination of SOCS box was not necessary for exosomal transport of SPSB1. Considering the ability of SOCS box proteins in inducing the formation of active ubiquitin ligase complex, it is possible that SOCS box recruits ubiquitin ligase complex and causes the ubiquitination of SPRY domain, which serves as the signalling for SPSB1's sorting into MVBs and exosomes. Furthermore, the tetraspannin CD63, a late endosome/ MVB marker protein, has been found to associate with LMP1 and mediate its exosome secretion [4]. Our immunofluorescence data showed the recruitment of SPSB1 into CD63-postive late endosome by Ras (Fig. 5), indicating that SPSB1 shares endosomal-exosomal trafficking with CD63.

Previous study has revealed the biological effects of exosomes on the recipient cells. For example, PTEN carried on exosomes are able to inhibit proliferation of recipient cell by suppressing PI3K/AKT signalling [19]. In this study, we showed that the SPSB1- and Ras-containing exosomes were readily to be internalized by recipient cells (Fig. 8). It remains to be seen how exosomal SPSB1 and Ras may contribute to TGF-β signalling in recipient cells.

Material and Methods

DNA constructs and primers

v-Ha-Ras was cloned into the pcDNA3 mammalian cell expression vector [21] and FLAG-SPSB1, MYC-SPSB1, MYC-SPSB1Δ, MYC-SPSB1(Y129A) were cloned into the pEF-BOS mammalian cell expression vector as previously described [22]. MYC-SPSB1 (K267R) was generated based on MYC-SPSB1 using Quick Change® II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's recommendations. Primers used for site-direct mutagenesis were: MYC-SPSB1 (K267R) forward: GAGGTAGGCTCTGAGGGAGGCAGGTAGCGG. MYC-SPSB1 (K267R) reverse: GAGGTAGGCTCTGAGGGAGGCAGGTAGCGG. All newly generated constructs were verified by sequencing.

Antibodies and reagents

The following antibodies were used: anti-FLAG (M2) antibody (F3165, 1:5000 for WB and 1:2000 for IF) from Sigma-Aldrich; anti-cMYC antibody (sc-40, 1:1000 for WB) from Santa Cruz Biotechnology; anti-Ras antibody (610001, 1:1000 for WB and IF) from BD; anti-TSG101 antibody (612696, 1:1000 for WB) from BD; anti-Alix antibody (2127, 1:1000 for WB) from Cell Signalling; anti-CD81 antibody (sc-7637, 1:200 for WB) and anti-CD63 antibody (sc-5275, 1:500 for IF) from Santa Cruz Biotechnology; anti-β-Actin antibody (A2066, 1:5000 for WB) from Sigma-Aldrich. Rabbit polyclonal anti-SPSB1 antibody (1:500 for IF) was generated in house. HRP-conjugated secondary antibodies (1:1000) were purchased from Bio-Rad. The Alexa Fluor® 488- and Alexa Fluor® 546-conjugated secondary antibodies (1:500) used for immunofluorescence staining were purchased from Invitrogen.

Hoechst (H6024) was purchased from Sigma-Aldrich. Monensin (M5273), wortmannin (W1628), GW4869 (D1692), Brefeldin A (B7651), 5-(N,N-Dimethyl) amiloride hydrochloride (DMA) (A4562) were purchased from Sigma-Aldrich.

Cell lines, cell culture and transfection

Human breast cancer cell line MDA-MB-231 and human embryonic kidney cell line HEK 293T were cultured in DMEM (Gibco, Life technology) supplemented with 10% fetal calf serum, 10 μg/mL penicillin and 100 μg/mL streptomycin (Invitrogen) at 37 °C with 10% CO_2

in a humidified atmosphere. HEK293T and MDA-MB-231 were originally obtained from ATCC (American Type Culture Collection). HEK 293T cells were transiently transfected with indicated DNA constructs using FuGENE HD transfection reagent (Promega).

Exosome isolation

Exosomes were isolated by differential centrifugation as previously described with slight modifications [11]. Briefly, HEK 293T cells (4×10^5-5×10^7) transfected with indicated DNA constructs were grown for 48h before cells were cultured in bovine exosomes-depleted medium (DMEM containing 1% FCS and 1% penicillin-streptomycin was centrifuged overnight at 100,000 × g to deplete bovine exosomes) or serum free medium for 24h. The culture media were harvested and subjected to sequential centrifugation steps (300 × g for 5min, 2000 × g for 15 min). The supernatants were further concentrated by 100K NMWL centrifugal filtration (Amicon Ultra-15, Millipore) and washed twice with 1× PBS before centrifuged at 10,000 × g for 90min. Exosomes were recovered from the cleared, condensed supernatant by ultracentrifugation at 100,000 × g for 17h at 4 °C. Exosome pellet was resuspended in ice-cold 1 × PBS and stored at 4°C. Exosomes used for Western blotting analysis were resuspended in 1 × Laemmli Sample Buffer (Bio-Rad) before application. Exosomal protein amount was determined by BCA Protein Assay Kit (Thermo Scientific).

Cryo-TEM analysis

Cryo-transmission electron microscopy (cryo-TEM) analysis of exosomes was performed as previously described [23]. Briefly, isolated exosomes were resuspended in ice-cold 1× PBS and transferred onto glow-discharged C-flat holey carbon grids. Excess liquid was blotted and grids were frozen and mounted in a Gatan cryoholder in liquid nitrogen. The grids were transferred to Tecnai F30 (FEI) electron microscope at liquid nitrogen temperature and the samples were observed at 300 kV. Representative images were presented.

Western blotting analysis

Cells were lysed using lysis buffer (30mM HEPES, 1% TritonX-100, 2mM MgCl, 150mM NaCl, 5mM EDTA, complete protease inhibitor tablet and phosphostop phosphatase cocktail tablet) on ice for 30min. The cell debris was removed by centrifugation at 13000 rpm for 15 min. The lysate was heated at 95 °C for 10 minutes in 1 × Laemmli sample buffer (Bio-Rad). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare). After blocking with 4% skim milk for 1h at room temperature (RT), the

membranes were probed with indicated primary antibody and corresponding secondary antibody. The signal was visualized by using the Western Lightning® ECL Pro Enhanced Chemiluminescence Substrate (PerkinElmer). All Western blotting results were representative of three separate experiments.

Immunofluorescence staining and confocal microscopy

HEK 293T cells (4×10^5) were grown on glass cover slips and transfected with indicated DNA constructs for 48h. Cells were fixed in 3.7% formaldehyde in PBS (Sigma-Aldrich) for 10 min at RT. After washing 3 times with 1×PBS, cells were permeabilized with 0.1% Triton-X-100 (Merck) for 5 min at RT and washed 3 times with 1× PBS. Cells were then blocked with PBS containing 5% BSA for 1 h at RT. Thereafter, cells were probed with indicated primary antibodies for 2h at RT. After washing with 1× PBS for 3 times, cells were incubated with Alexa Fluor® 488- or Alexa Fluor® 546-conjugated secondary antibodies for 1h at RT and were then washed 3 times with 1× PBS. Cell nuclei were stained with Hoechst stain for 10 minutes at RT. Following three times 1× PBS wash and one time double-distilled water (DDW) wash, HEK 293T cells were mounted onto a glass slide using Prolong Gold antifade reagent (Invitrogen). Images were collected by Olympus FV1000 confocal microscope (magnification = 60×) and processed using Olympus Fluroview Ver. 1.7c. Representative images were presented.

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Figure Legends

Figure 1. Ras reduces the cellular pool of SPSB1 by mediating SPSB1's export via exosomes. **(A)** Differential centrifugation protocol for isolating exosomes from cell culture media. HEK 293T cells (2×10^7) were transfected with plasmid encoding FLAG-SPSB1 alone or in combination with plasmid encoding Ras. After 48h, cells were cultured in bovine exosomes-depleted medium (DMEM containing 1% FCS and 1% penicillin-streptomycin was centrifuged at 100,000 × g for 16h at 4°C to deplete bovine exosomes) for further 24h for exosome production. Exosomes were isolated according to the differential centrifugation protocol. **(B)** P100 fraction was subjected to cryo-TEM and Western blotting analysis (30μg) for exosome characterization. Scale bar, 100nm. **(C)** Cells and exosomes were lysed and analysed for the expression of SPSB1, Ras, β-Actin and exosome marker TSG101 by Western blotting. Molecular mass markers (in kilo Daltons) were shown to the left. Data are representative of three experiments.

Figure 2. Ras-mediated exosome export of SPSB1 is blocked by exosome secretion inhibitors whereas promoted by enhancer. **(A)** HEK 293T cells (2×10^7) were transfected with plasmid encoding FLAG-SPSB1 in combination with plasmid encoding Ras. After 48h, cells were cultured in bovine exosomes-depleted medium in the presence of indicated doses of Monensin for further 16h. Exosomes were isolated according to the differential centrifugation protocol as outlined in Fig. 1A. Cells and exosomes were lysed and analysed for the expression of SPSB1, Ras, β-Actin and exosome marker TSG101 by Western blotting. **(B)** HEK 293T cells (4×10^5) were transfected with plasmid encoding FLAG-SPSB1 alone. After 48h, cells were treated with indicated doses of Monensin for16h before they were lysed and analysed by Western blotting. **(C, D)** HEK 293T cells (4×10^5) were transfected with plasmid encoding FLAG-SPSB1 in combination with plasmid encoding Ras. After 48h, cells were cultured in serum free medium in the presence of 25nm DMA **(C),** 10μM GW4869 or 160nm wortmannin **(D)** for 16h before their culture media was collected. Cells and exosomes (exosomes enriched fraction: supernatant III in Fig. 1A) were analysed the same way as above. Data are representative of three experiments.

Figure 3. SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain is required for SPSB1's exosome secretion. **(A)** A schematic representation of SPSB1-129 and SPSB1Δ mutants. SPRY domain, SOCS box and mutation site (Y129A) were indicated. **(B)** HEK 293T cells (4×10^5) were transfected with indicated DNA constructs.

After 48h, cells were cultured in serum-free medium for further 16h for exosome production. Cells and exosomes (exosomes enriched fraction) were analysed the same way as above. Data are representative of three experiments.

Figure 4. Ras expression causes the accumulation of SPSB1 in perinuclear vesicular structures. HEK 293T cells (4×10^5) were transfected with indicated DNA constructs. 48h post-transfection, cells were fixed and processed for immunofluorescence staining with antibodies against SPSB1 (rabbit, 1:500, green) and Ras (mouse, 1:1000, red). Images were collected by Olympus FV1000 confocal microscope and processed using the FV1000 software (magnification = 60×). Merged fluorescence images (yellow) indicated the co-localization of indicated proteins. Scale bar, 10 μm. Representative images were presented.

Figure 5. SPSB1 localizes to CD63-positive late endosomes/MVBs when Ras is overexpressed. HEK 293T cells (4×10^5) were transfected with indicated DNA constructs. 48h post-transfection, cells were fixed and processed for immunofluorescence staining with antibodies against SPSB1 (rabbit, 1:500, green) and CD63 (mouse, 1:500, red). Nuclei were stained with Hochest (blue). Images were collected by Olympus FV1000 confocal microscope and processed using the FV1000 software (magnification = 60×). Merged fluorescence images (yellow) indicated the co-localization of indicated proteins. Insets are magnified images of the boxed region. Scale bar, 10 μm. Representative images were presented.

Figure 6. The ubiquitination of SOCS box is not responsible for Ras-induced recruitment of SPSB1 into exosomes. **(A)** Schematic representation of SPSB1-267 mutant with SOCS box K-to-R change. Electropherograms confirmed that the nucleotide of AAA codon (Lys) was replaced by a G nucleotide after site-directed mutagenesis. **(B)** HEK 293T cells were transfected with indicated DNA constructs. 48h post-transfection, cells were cultured in serum free medium for 16h for exosome production. Cells and exosomes (exosomes enriched fraction) were analysed the same way as above. Data are representative of three experiments. **(C)** HEK 293T cells were transfected with indicated DNA constructs. 48h post-transfection, cells were fixed and processed for immunofluorescence staining with antibodies against SPSB1 (rabbit, 1:500, green) and Ras (mouse, 1:500, red). Images were collected by Olympus FV1000 confocal microscope and processed using the FV1000 software (magnification = 60×). Merged fluorescence images (yellow) indicated the co-localization of indicated proteins. Scale bar, 10 μm. Data are representative of three experiments.

Figure 7. SPSB1-267 mutant accumulates in CD63-positive late endosomes/MVBs upon Ras co-expression. HEK 293T cells were transfected with indicated DNA constructs. 48h post-transfection, cells were fixed and processed for immunofluorescence staining with antibodies against SPSB1 (rabbit, 1:500, green) and CD63 (mouse, 1:500, red). Nuclei were stained with Hochest (blue). Images were collected by Olympus FV1000 confocal microscope and processed using the FV1000 software (magnification = 60×). Merged fluorescence images (yellow) indicated the co-localization of indicated proteins. Scale bar, 10 μm. Representative images were presented.

Figure 8. Uptake of SPSB1- and Ras- containing exosomes by recipient MDA-MB-231 cells. Exosomes were harvested from 5×10^7 untransfected HEK 293T cells or HEK 293T cells transiently co-transfected with plasmid encoding FLAG-SPSB1 and plasmid encoding Ras (v-Ha-Ras). MDA-MB-231 cells were incubated with indicated exosomes (20μg) for 1-2h. MDA-MB-231 cells were fixed and processed for immunofluorescence staining with antibodies against FLAG-SPSB1 (anti-FLAG, mouse, 1:2000, green) or Ras (mouse, 1:1000, red). Nuclei were stained with Hochest (blue). Images were collected by Olympus FV1000 confocal microscope and processed using the FV1000 software (magnification = 60×). Uptake of exosomal SPSB1 (green) and Ras (red) (indicated by white arrows) by recipient MDA-MB-231 cells were observed. Scale bar, 10 μm. Representative images were presented.

Figure 9. Proposed model for Ras-dependent exosomal release of cytosolic protein SPSB1. The cytosolic protein SPSB1 down-regulates TGF-β signalling through targeting TβRII to proteasome for degradation. SPSB1 is secreted in association with exosomes upon Ras overexpression, triggering a reduction in the cellular pool of SPSB1. Ras-mediated SPSB1's MVB sorting and exosome secretion are dependent on ceramide, PI3K and intracellular Ca^{2+} concentration. SPSB1's SOCS box as well as the interaction between Ras and SPSB1's SPRY domain controls SPSB1's co-localization with CD63-positive late endosome and exosome release, while the ubiquitination of SOCS box is not required. Moreover, the SPSB1- and Ras- containing exosomes can be internalized by recipient cells.

Figures

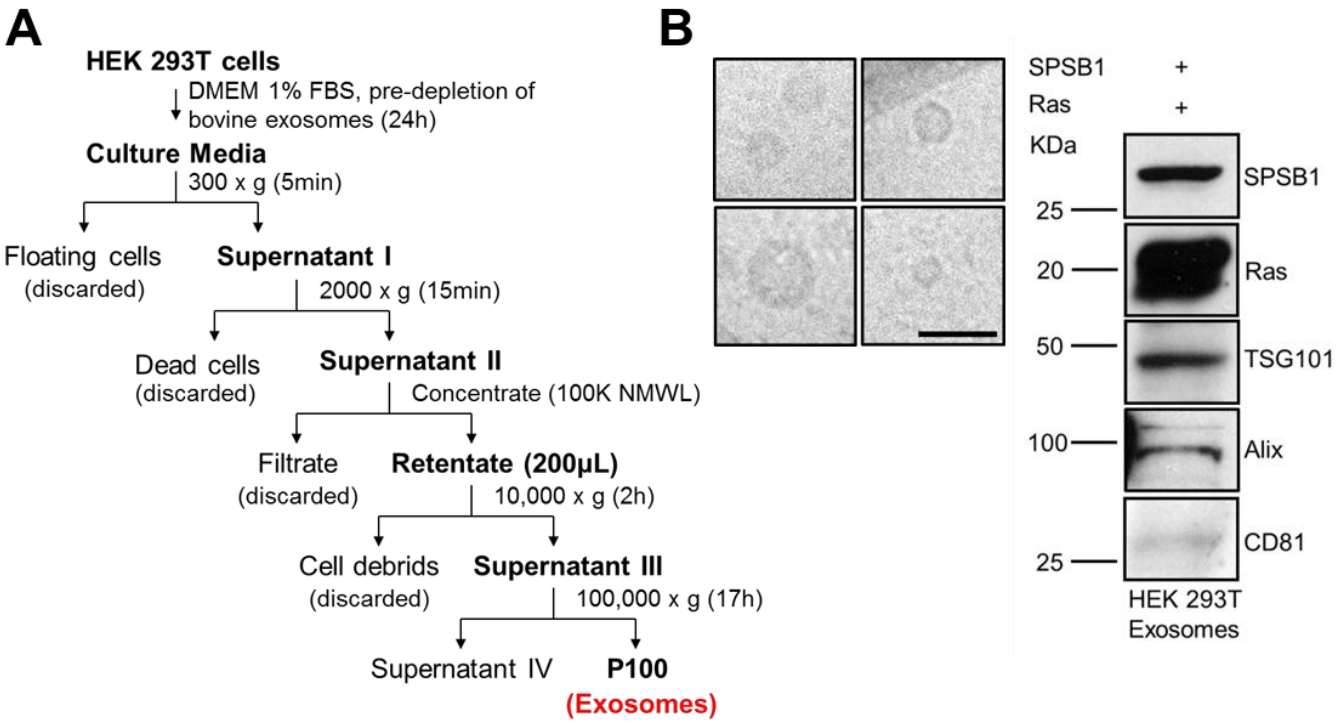
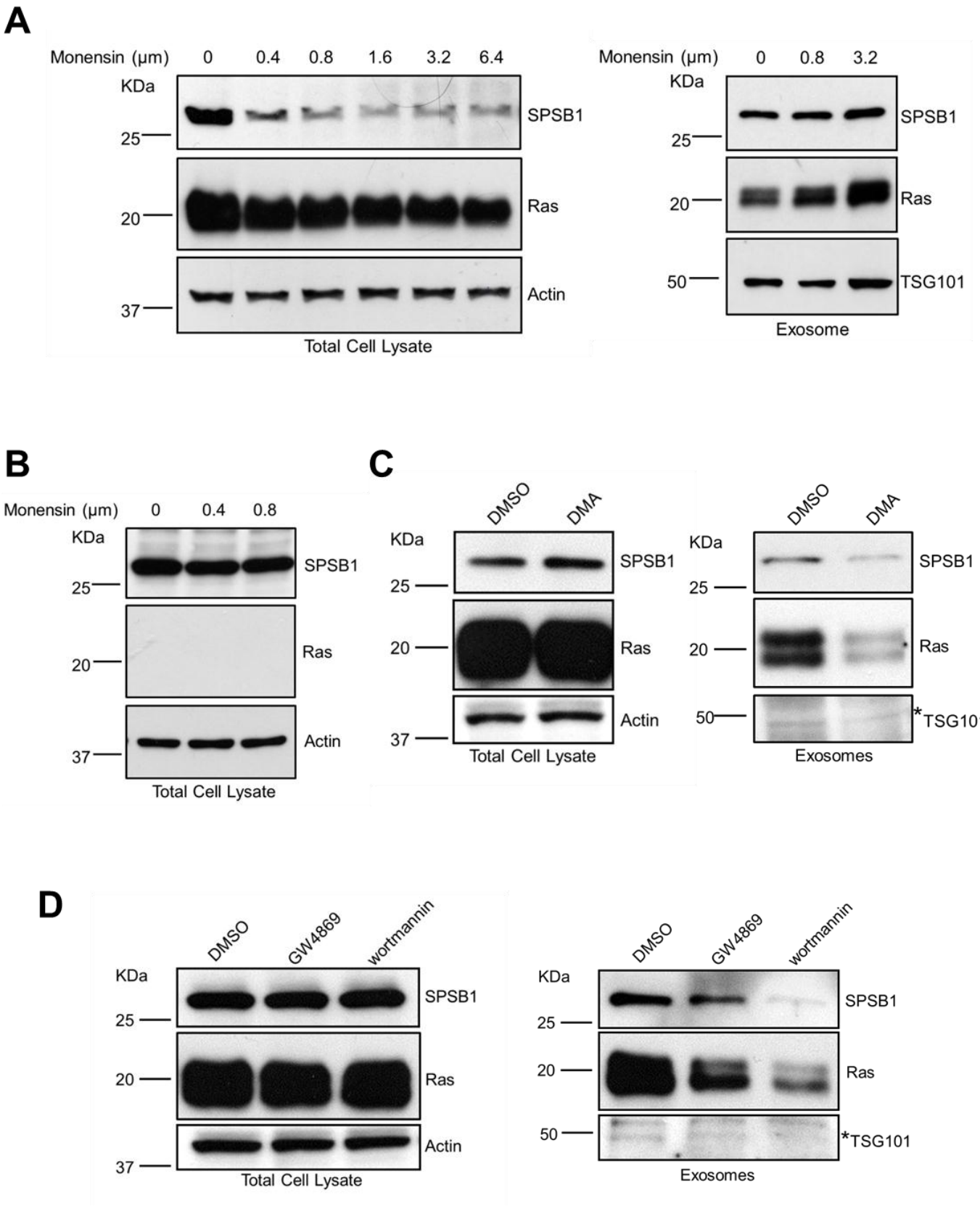


Figure 1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KDa
25
20
37
SPSB1
Ras
Actin
Total Cell Lysate
Monensin (μm) 0 0.8 3.2
KDa
25
20
50
SPSB1
Ras
TSG101
Exosome
B
Monensin (μm) 0 0.4 0.8
KDa
25
20
37
SPSB1
Ras
Actin
Total Cell Lysate
C
DMSO
DMA
KDa
25
20
37
SPSB1
Ras
Actin
Total Cell Lysate
DMSO
DMA
KDa
25
20
50
SPSB1
Ras
*TSG101
Exosomes
D
DMSO
GW4869
wortmannin
KDa
25
20
37
SPSB1
Ras
Actin
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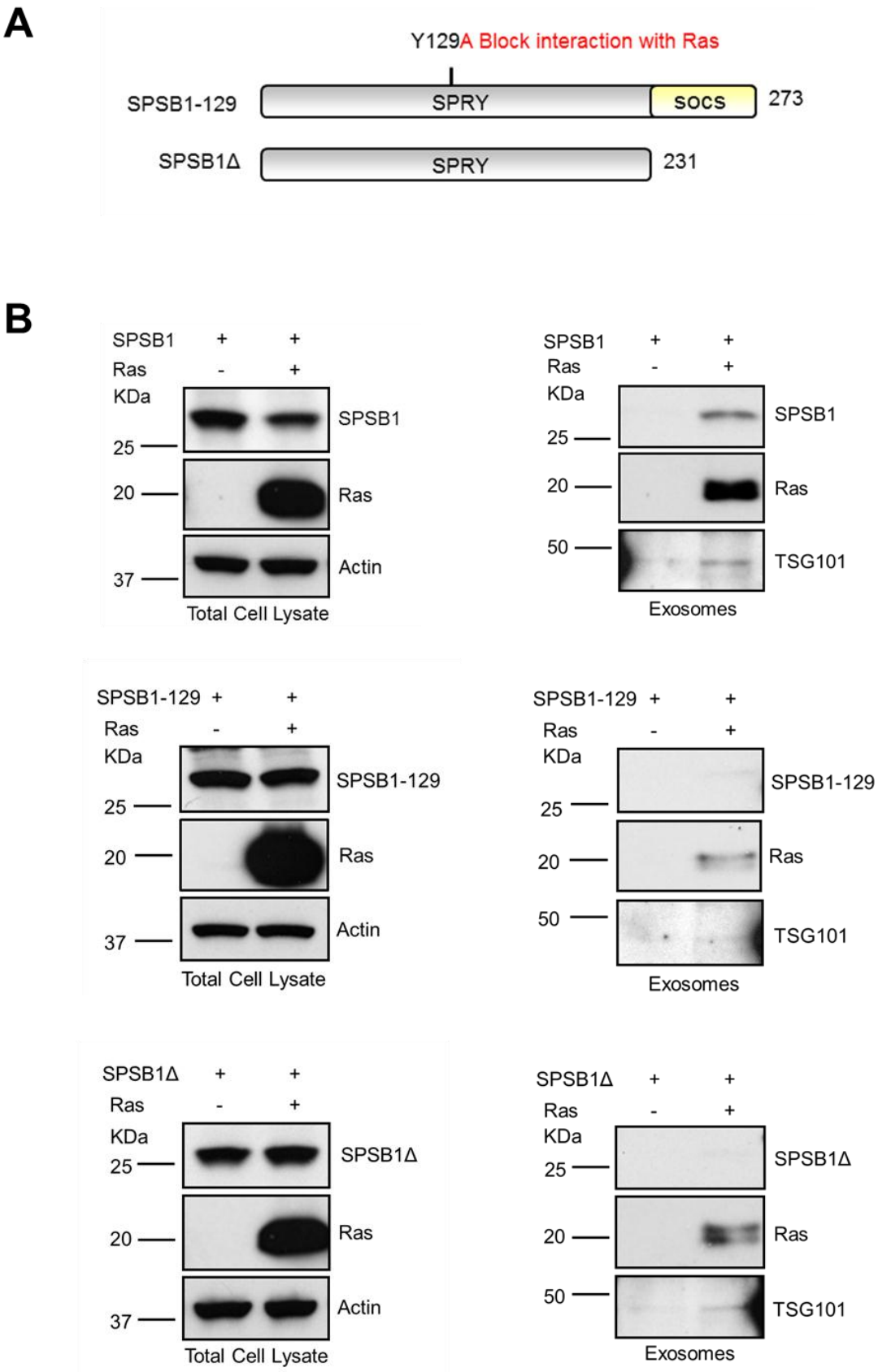
SPSB1
Ras
*TSG101
Exosomes

Figure 2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SOCS
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Ras - +
KDa
25
20
37
SPSB1
Ras
Actin
Total Cell Lysate
SPSB1 + +
Ras - +
KDa
25
20
50
SPSB1
Ras
TSG101
Exosomes
SPSB1-129 + +
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20
37
SPSB1-129
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Ras - +
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Ras - +
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Ras
TSG101
Exosomes

Figure 3

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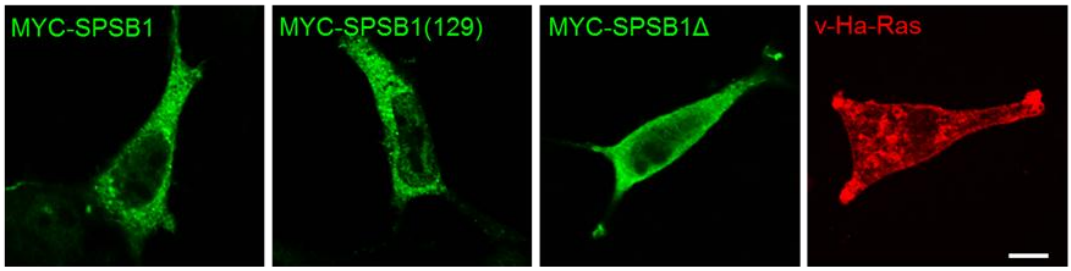
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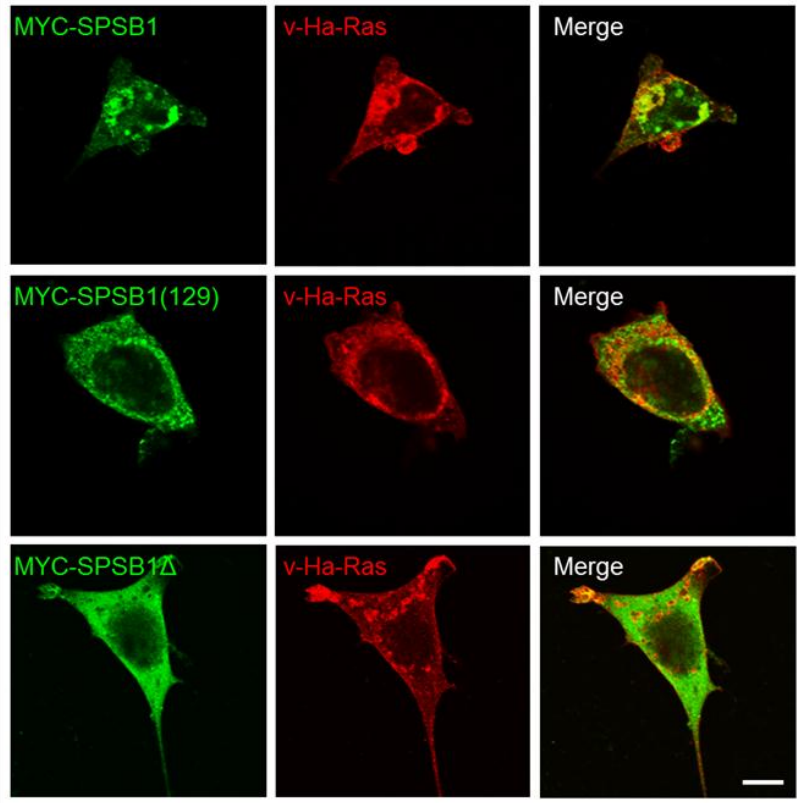
v-Ha-Ras

B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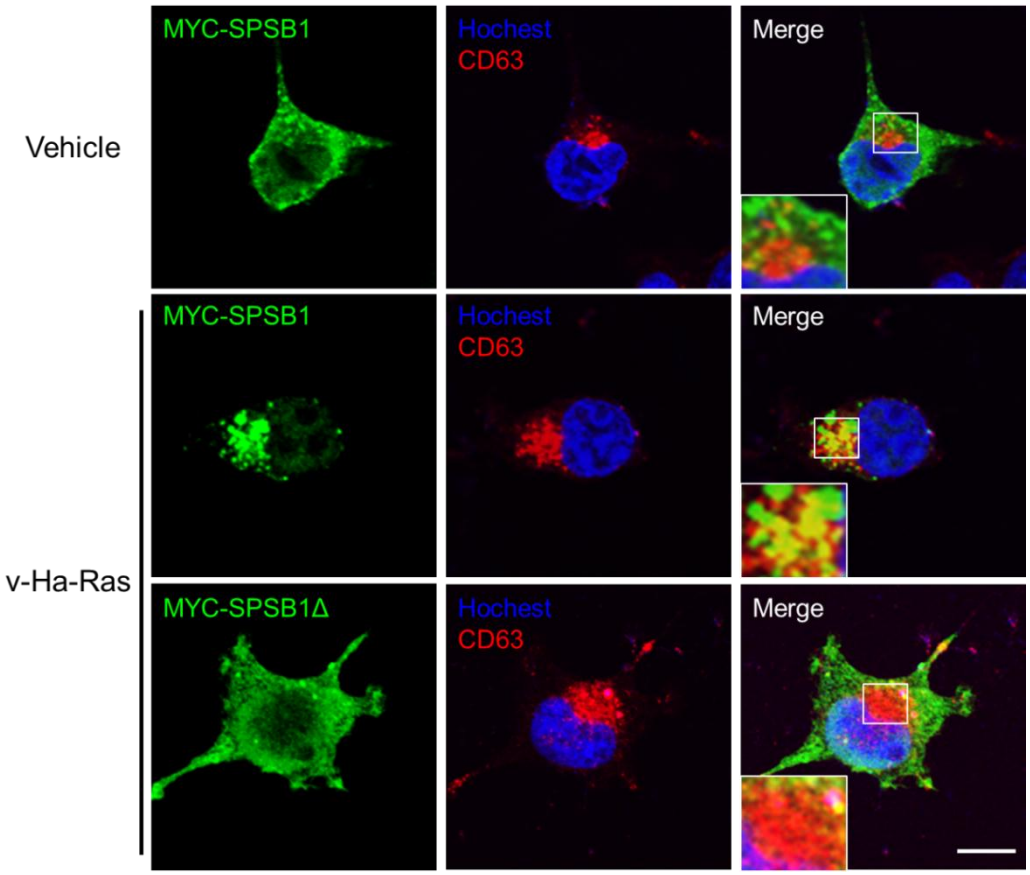
MYC-SPSB1(129)
v-Ha-Ras
Merge
MYC-SPSB1Δ
v-Ha-Ras
Merge

Figure 4


Vehicle
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MYC-SPSB1
Hochest
CD63
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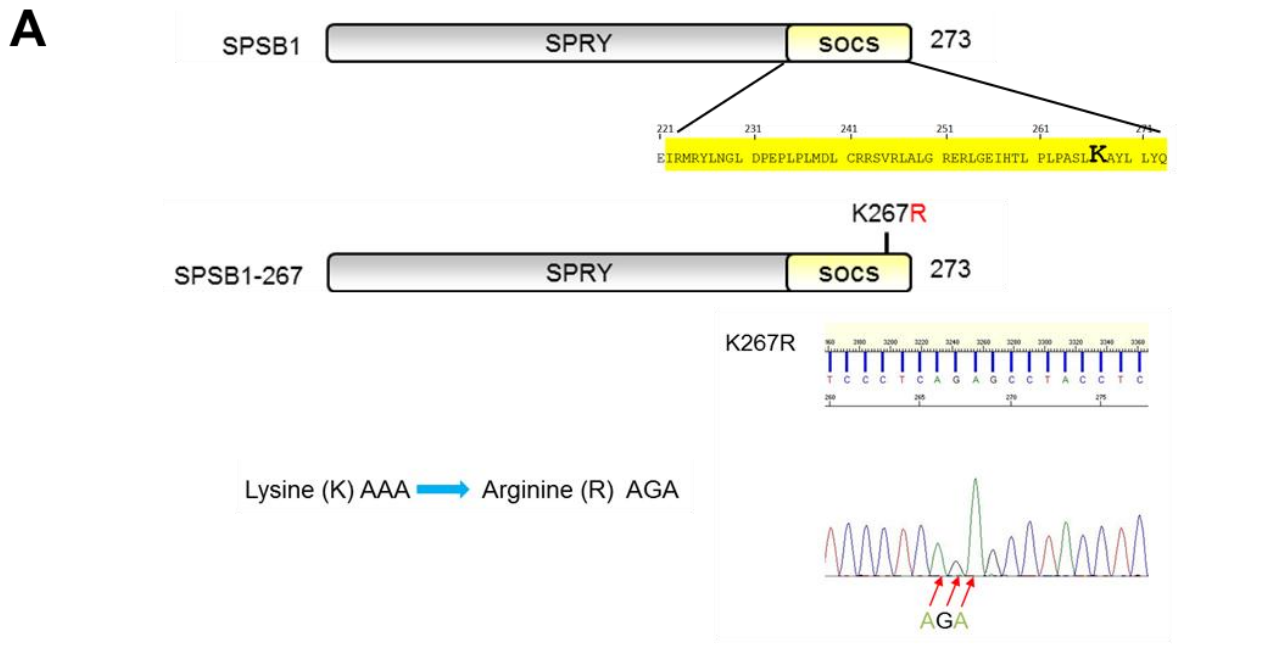
Hochest
CD63
Merge
MYC-SPSB1Δ
Hochest
CD63
Merge

Figure 5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K267R
SPSB1-267
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K267R
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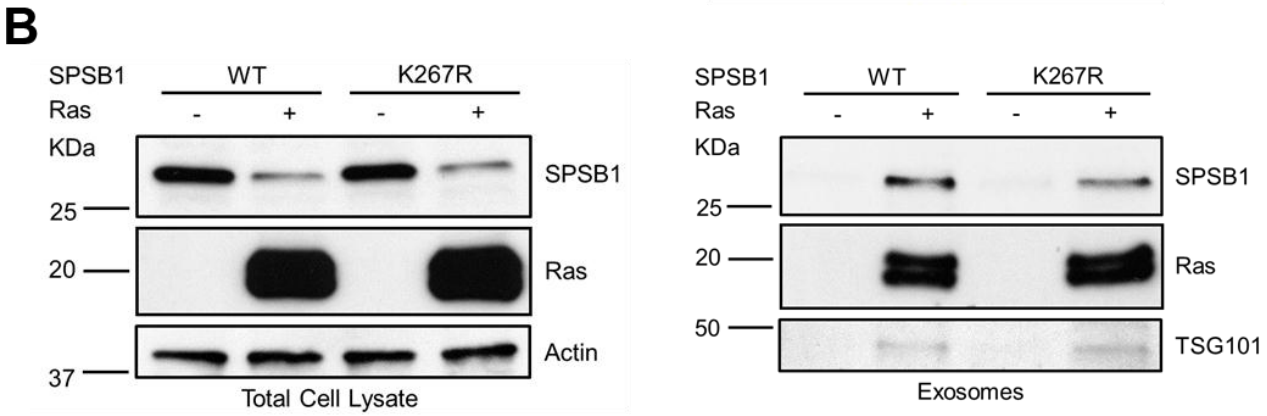
Lysine (K) AAA
Arginine (R) AGA
AGA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Ras
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Actin
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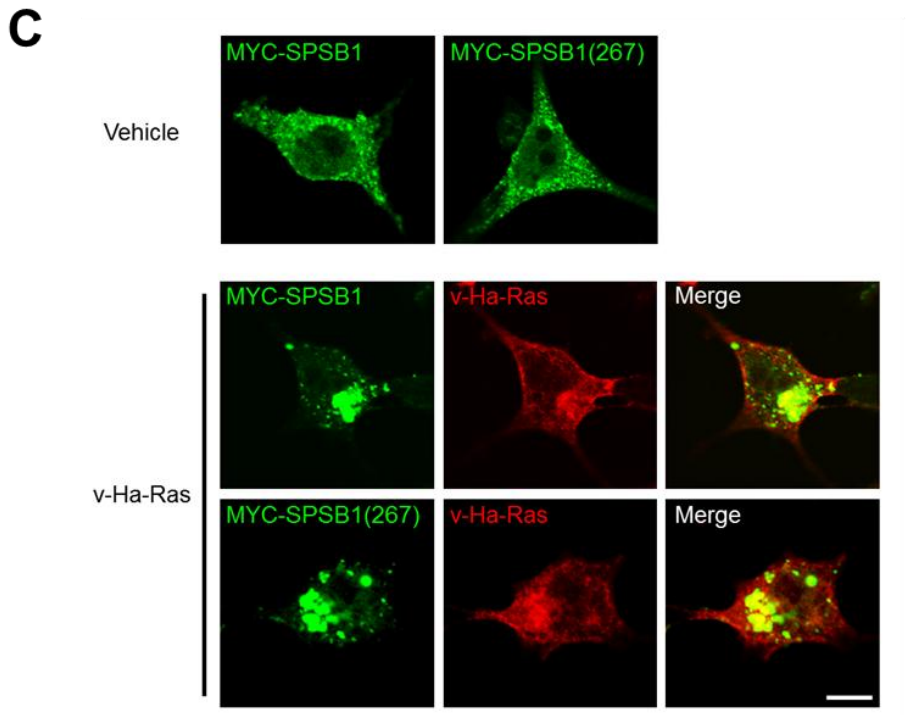
Exosomes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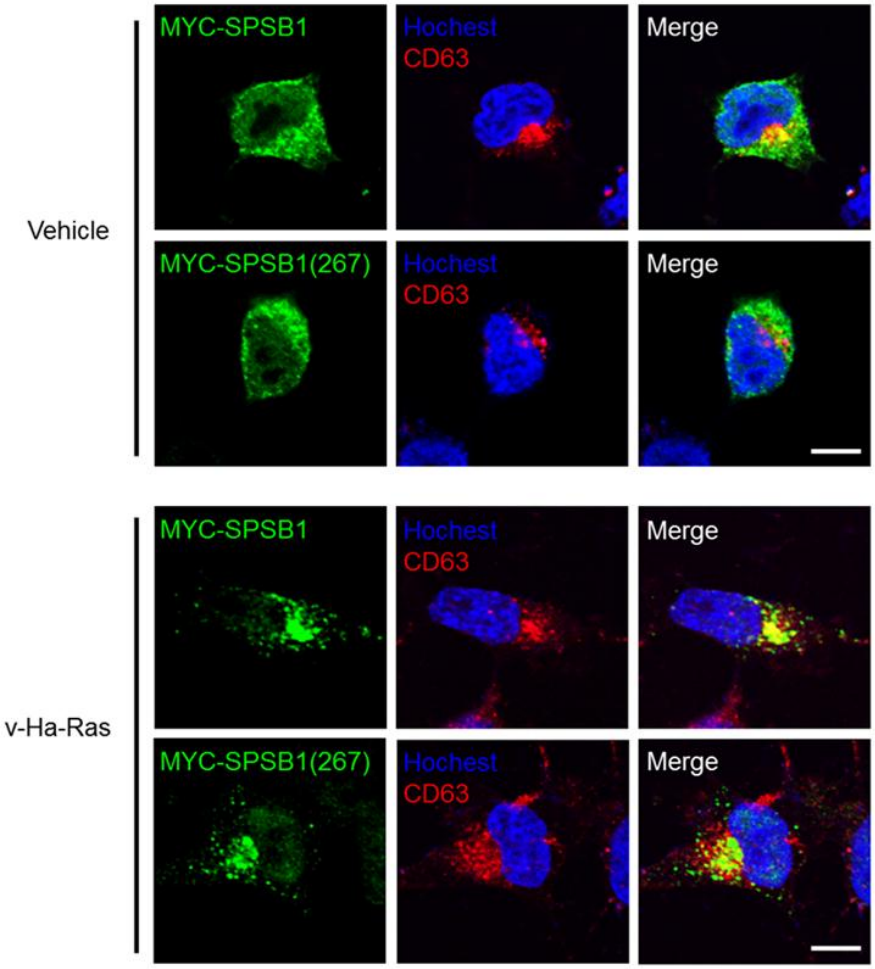
v-Ha-Ras
Merge

Figure 6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Hochest
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CD63
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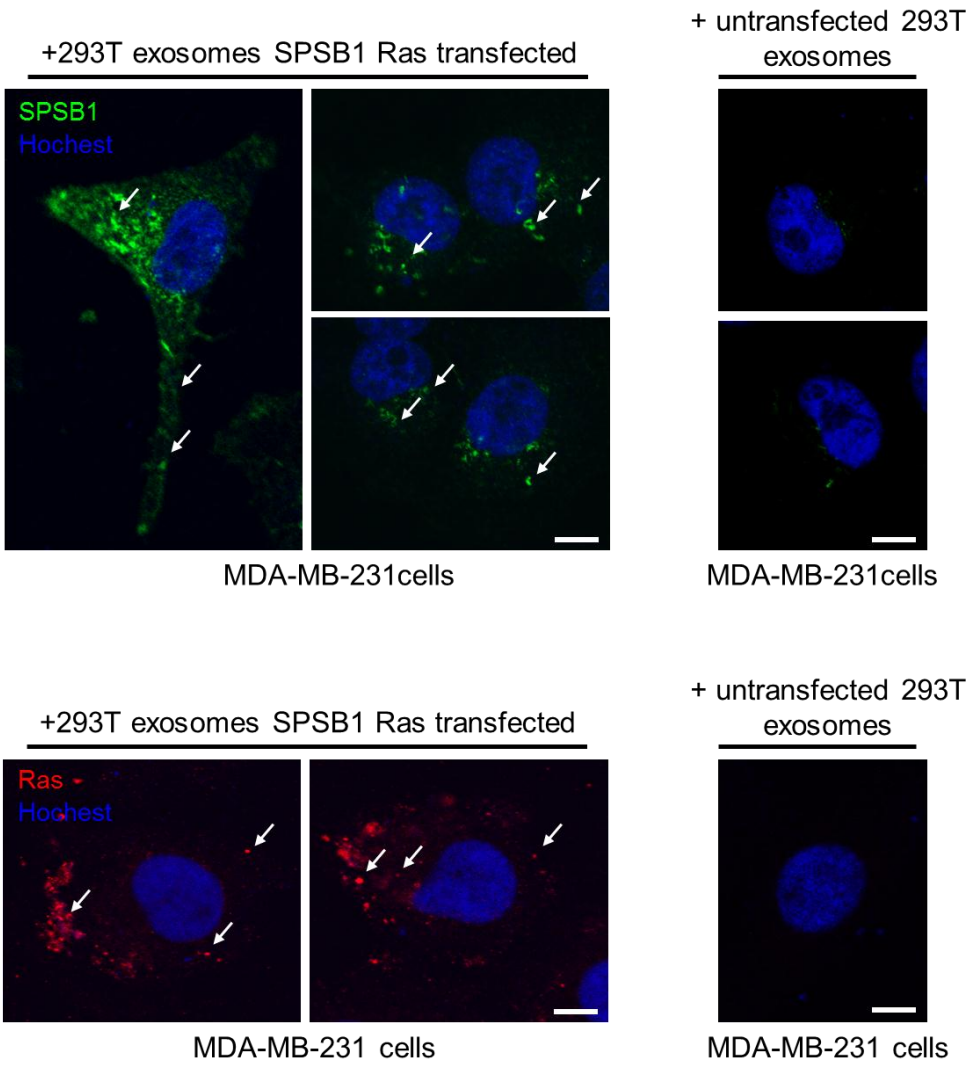
Figure 7


+293T exosomes SPSB1 Ras transfected
+ untransfected 293T exosomes
SPSB1
Hochest
MDA-MB-231cells
MDA-MB-231cells
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Ras
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MDA-MB-231 cells
MDA-MB-231 cells

Figure 8

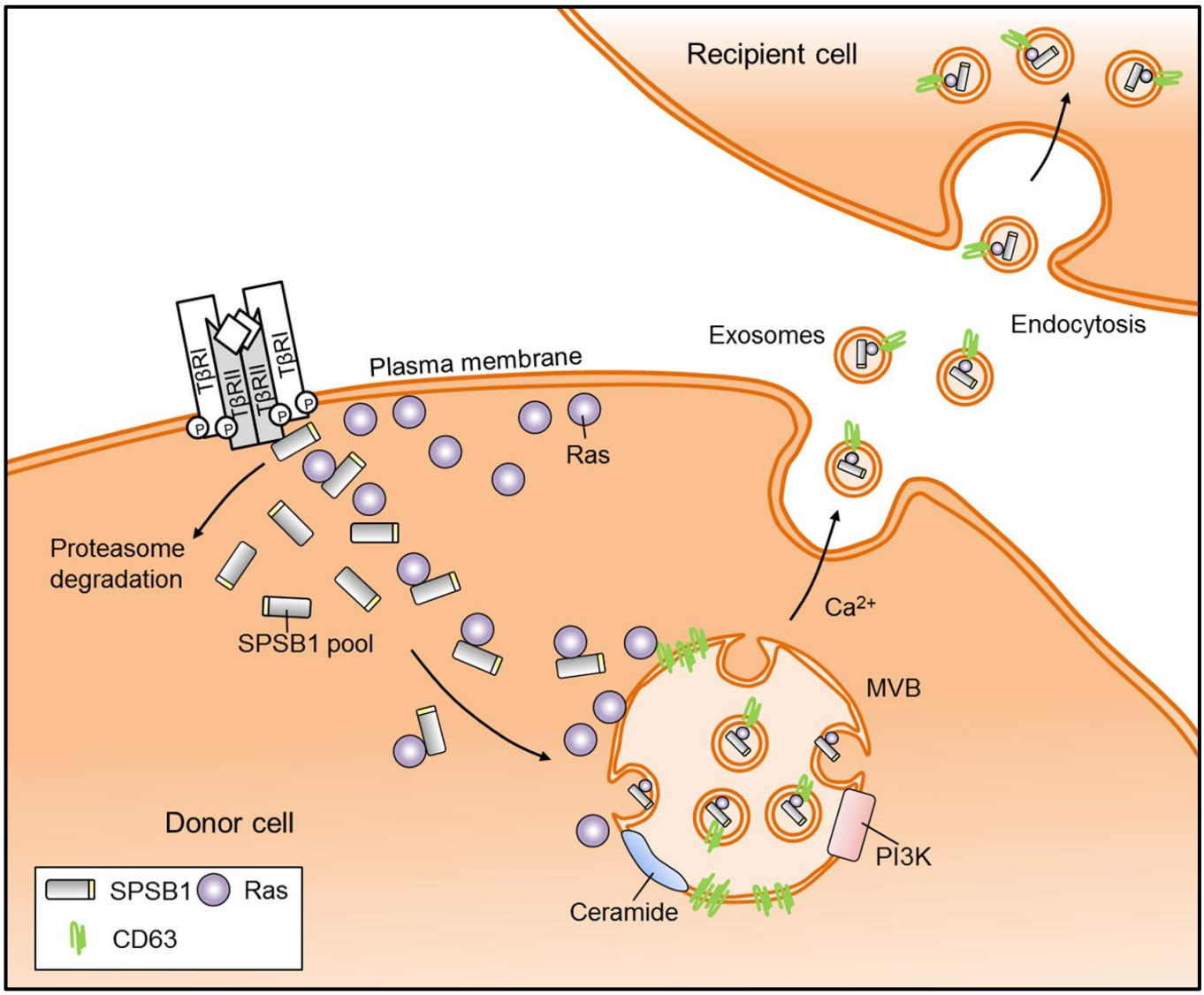
Recipient cell
Endocytosis
Exosomes
TβRI
TβRII
TβRIII
TβRI
Plasma membrane
Ras
Proteasome
degradation
SPSB1 pool
Ca2+
MVB
Donor cell
PI3K
Ceramide
SPSB1
Ras
CD63

Figure 9

Chapter 5. Ras expression increases the exosomal TGF-β secretion

Introduction

RAS (*HRAS, KRAS* and *NRAS*) are well recognized as the most frequently mutated oncogenes in human cancers [233]. The mutations in *RAS* genes lock Ras in the GTP-bound form which is insensitive to GAP, conferring them oncogenic property. Ras proteins appear as pivotal signalling transducers that modulate multiple biological processes, including proliferation, invasion and survival. Ras proteins have been shown to recruit and activate PI3K in endosome [36], which is known to drive MVBs and exosome biogenesis [37, 38]. In particular, Ras signalling has been revealed to cooperate with TGF-β signalling during cancer progression. Ras signalling synergizes with TGF-β signalling by enhancing tumorigenic effects of TGF-β. Sustained activation of Ras resulted in epithelial-mesenchymal transition, which was maintained by Ras signalling-mediated autocrine loop of TGF-β. The mesenchymal phenotype was reversed upon the removal of autocrine TGF-β [242, 243]. Thus, it is possible that Ras expression can also enhance the release of exosomal TGF-β, thus contributing to EMT-associated cancer development. In the present study, we investigate whether Ras expression can cause alterations in the levels of exosomal TGF-β.

Results

5.1 Exosomes derived from the Ras-transformed MDCK cells (21D1 cells) induce higher TGF-β signalling activity than that from MDCK cells.

To assess the contribution of Ras to exosomal TGF-β levels, exosomes were isolated from MDCK cells and the Ras-transformed MDCK (21D1) cells by differential centrifugation as outlined in Fig. 1A of Chapter 3. 21D1 cells exhibited a spindle-like phenotype while MDCK cells displayed a cobblestone-shapped morphology [119] (Fig. 5.1A). Addition of 10,000 × g supernatant (supernatant III in Fig. 1A of Chapter 3) from 21D1 cells resulted in 20-fold of induction of TGF-β activity as compared with basal reporter level, while no induction was observed after addition of 10,000 × g supernatant from MDCK cells. TGF-β signalling-inducing activity were completely depleted in 100,000 × g supernatant (supernatant IV in Fig. 1A of Chapter 3) after exosomes were pelleted by ultracentrifugation, indicating that active TGF-β were in association with exosomes. This result also suggested that free-ligand TGF-β was cleared during differential centrifugation steps. Consistently, TGF-β signalling activity induced by exosomes from 21D1 cells was much higher than that from MDCK cells (Fig. 5.1B).

In line with these findings, increased levels of exosomal TGF-β were observed in 21D1 cells than MDCK cells as determined by TGF-β reporter activity assay after cell number or exosome mass normalization (Fig. 5.1C,D). Active TGF-β were significantly enriched in 21D1 exosomes but not in MDCK exosomes (Fig. 5.1D). In addition, the total amount of secreted exosomes was markedly elevated in 21D1 cells when compared with MDCK cells (Fig. 5.1E). Collectively, these data suggest that Ras expression enhances the production of exosomal TGF-β.

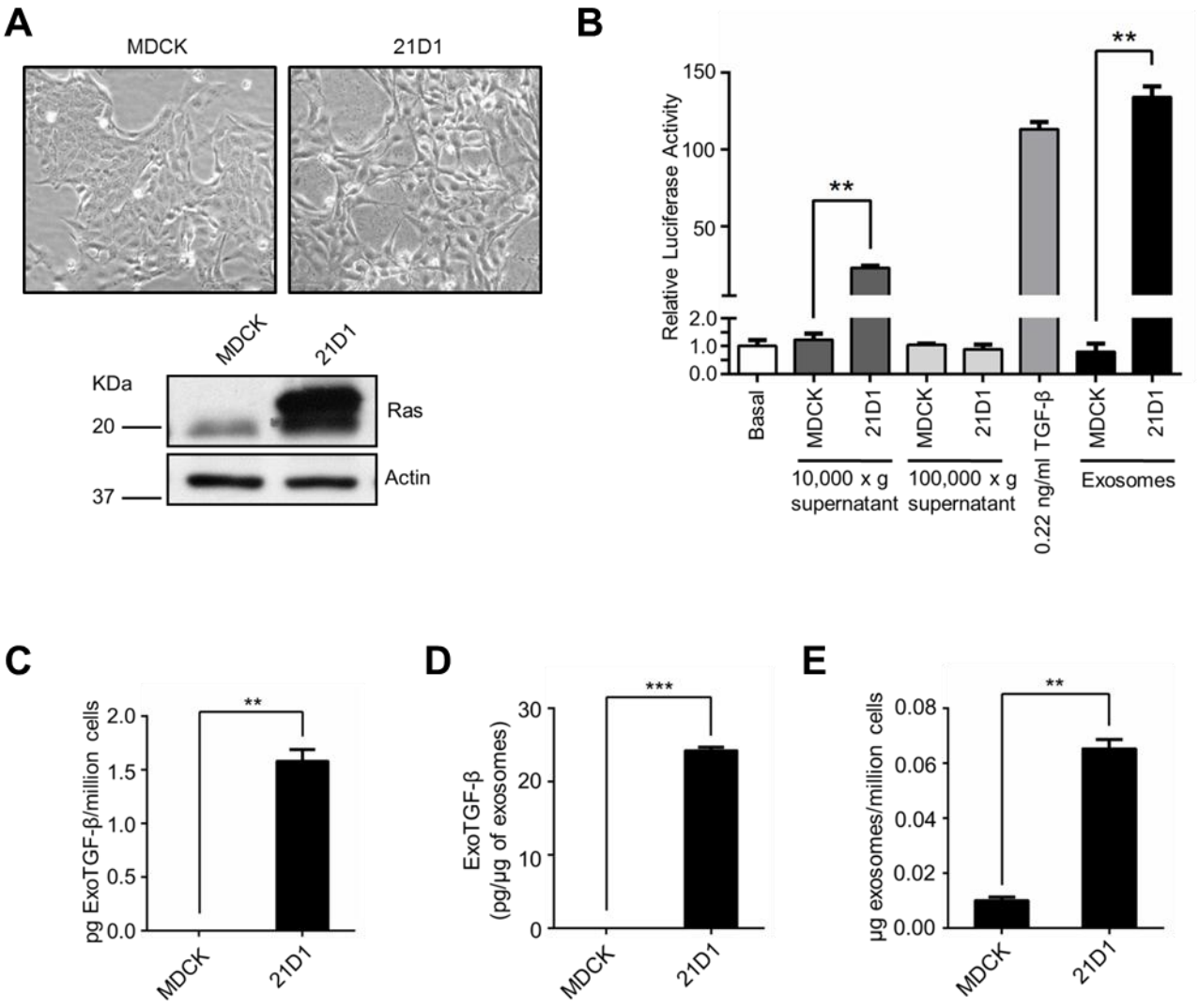

Figure 5.1. 21D1 cells have higher levels of exosomal TGF-β than MDCK cells. **(A)** Stable expression of Ras resulted in phenotypic transformation of MDCK cells (magnification=10 ×). MDCK cells and 21D1 cells were lysed and analysed by Western blotting for the expression of Ras and β-Actin. Molecular mass markers (in kilo Daltons) were shown to the left. **(B-E)** Exosomes were secreted by same numbers of MDCK cells and 21D1 cells (3×10^8). Exosomes were harvested from culture media of MDCK and 21D1 cells using differential centrifugation protocol as outlined in Fig. 1A of Chapter 3. The TGF-β reporter activities were measured in 21D1 reporter cells using the same way as in Fig. 1C of Chapter 3 and the exosomal TGF-β level was determined using standard curve generated by various concentrations of free-ligand TGF-β against the measured TGF-β reporter activities. The amounts of exosomes were quantified by BCA assay. Exosomal TGF-β levels were presented as per million cells (C) or per μg exosomes (D). Exosome amounts (μg) were presented as

per million cells (E). 10,000 × g supernatant was the supernatant after 10,000 × g centrifugation (supernatant III in Fig. 1A of Chapter 3). 100,000 × g supernatant was the supernatant after 100,000 × g centrifugation (supernatant IV in Fig. 1A of Chapter 3). Relative luciferase activity represents TGF-β reporter activity. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: **P <0.01, *** P <0.001.

5.2 Ras-transformed NIH3T3 cells display higher exosomal TGF-β levels as compared with normal NIH3T3 cells.

Next, we compared the exosomal TGF-β levels in Ras-transformed NIH3T3 (Ras- NIH3T3) cells and normal NIH3T3 (NIH3T3) cells. As shown in Fig. 5.2A, NIH3T3 cells grew in monolayer, whereas Ras-NIH3T3 cells lost contact inhibition and formed foci [261]. Immunoblotting confirmed the stable expression of Ras in Ras-transformed NIH3T3 cells. 8-fold induction of TGF-β activity was observed after addition of 10,000 × g supernatant from Ras-NIH3T3 cells, whereas 10,000 × g supernatant from NIH3T3 cells had low TGF-β signalling-inducing activity. Ultracentrifugation at 100,000 × g depleted TGF-β activity in the 100,000 × g supernatant, while higher TGF-β signalling activity was induced by addition of exosomes from Ras-NIH3T3 cells rather than that from NIH3T3 cells (Fig. 5.2B).

Consistently, after normalization by cell number and exosome mass, a marked increase in the levels of exosomal TGF-β was observed in Ras-NIH3T3 cells (Fig. 5.2C,D). Ras-transformation resulted in the high enrichment of active TGF-β in the exosomes (Fig. 5.2D) as well as a significant elevation in exosome production (Fig. 5.2E). Interestingly, unlike the exosomes from epithelial MDCK cells which had little TGF-β activity, the exosomes produced by NIH3T3 fibroblasts showed TGF-β activity.

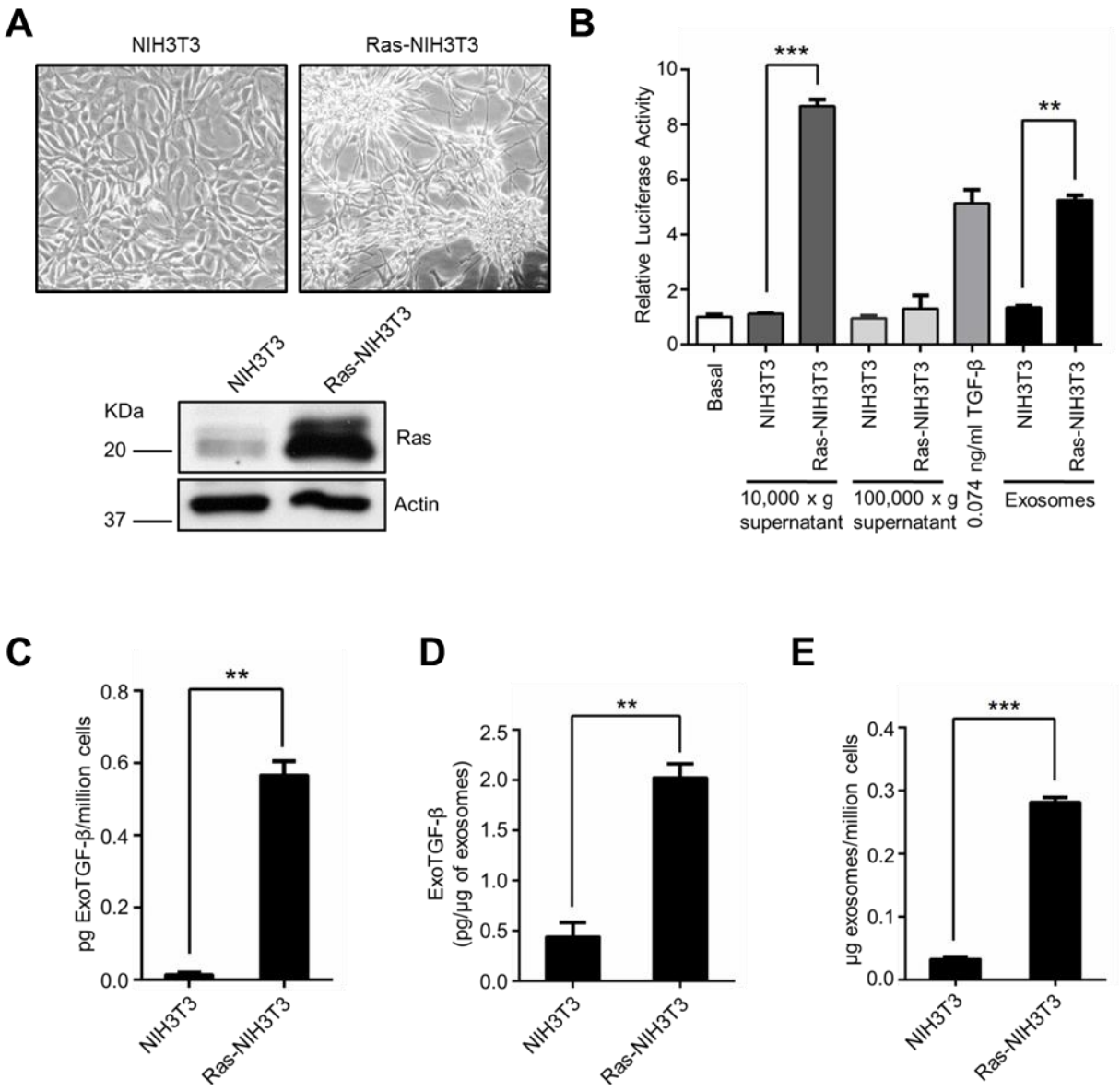

Figure 5.2. Exosomes secreted by Ras-transformed NIH3T3 cells induce higher TGF-β signalling than that from normal NIH3T3 cells. **(A)** Stable expression of Ras resulted in phenotypic transformation of NIH3T3 cells (magnification=10 ×). NIH3T3 cells and Ras-NIH3T3 cells were lysed and analysed by Western blotting for the expression of Ras and β-Actin. Molecular mass markers (in kilo Daltons) were shown to the left. **(B-E)** Exosomes were secreted by same numbers of NIH3T3 cells and Ras-NIH3T3 cells (1.5×10^8). Exosomes were harvested and the levels of exosomal TGF-β were determined the same ways as in Fig. 5.1. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: **P <0.01, *** P <0.001.

5.3 Expression of constitutively active H-Ras in HEK 293T cells results in elevated levels of exosomal TGF-β.

We next investigate whether the elevated exosomal TGF-β level is caused directly by Ras expression. Transient expression of v-Ha-Ras resulted in phenotypic transformation of HEK 293T cells (Fig. 5.3A). HEK 293T cells expressing constitutively active H-Ras mutants (Ras-293T) exhibited a round-up morphology [263]. Addition of exosomes from Ras-293T cells led to increased TGF-β reporter activity as compared with 293T exosomes which had no TGF-β signalling-inducing activity (Fig. 5.3B). Interestingly, no induction was observed after addition of 10,000 × g supernatant from either 293T cells or Ras-293T cells. These results suggested that exosomes from these cells had very low levels of active TGF-β.

As shown in Fig. 5.3C and Fig. 5.3D, Ras overexpression markedly increased the levels of exosomal TGF-β. More active TGF-β were carried in exosomes secreted by Ras-293T cells than exosomes from 293T cells (Fig. 5.3D), although the levels of exosomal TGF-β were significantly lower than that in 21D1 cells (Fig. 5.1D) and Ras-NIH3T3 cells (Fig. 5.2D). Also, Ras-293T cells secreted higher amounts of exosomes than 293T cells (Fig. 5.3E). Therefore, these data demonstrate the direct role of Ras in the production of exosomal TGF-β.

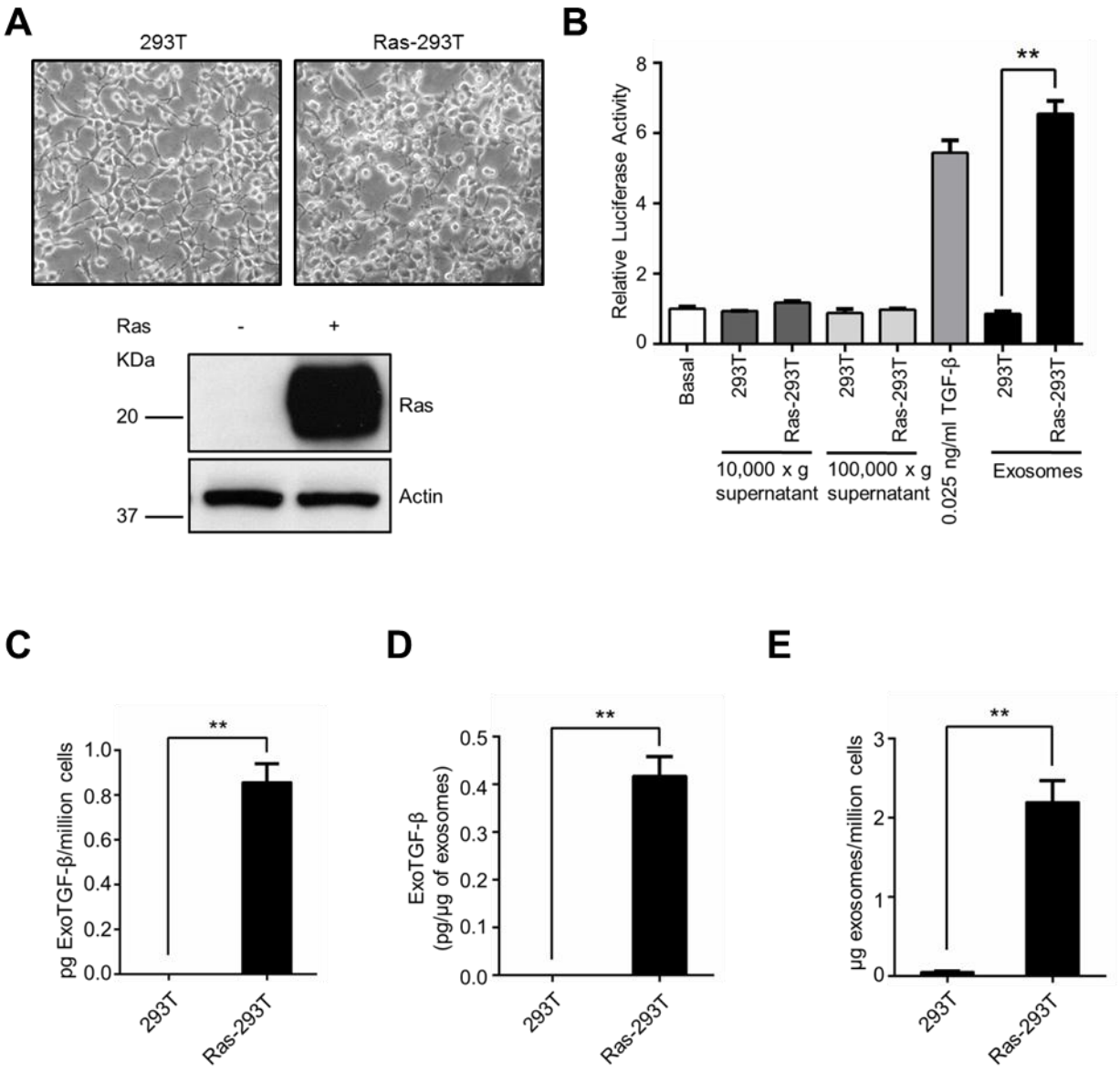

Figure 5.3. Transient expression of constitutively active H-Ras mutant in HEK 293T cells leads to enhanced production of exosomal TGF-β. **(A)** HEK 293T cells were transiently transfected with either pcDNA3 empty vector (293T) or v-Ha-Ras encoding plasmids (Ras-293T)**.** Transient expression of v-Ha-Ras resulted in phenotypic transformation of HEK 293T cells (magnification=10 ×). **(B-E)** Exosomes were secreted by same numbers of 293T cells and Ras-293T cells (1.65×10^7). Exosomes were harvested and the levels of exosomal TGF-β were determined the same ways as in Fig. 5.1. Data are representative of three experiments. Error bars are standard deviations (s.d) of triplicates. Significance level as indicated: **P <0.01.

Discussion

In this study, we report for the first time that Ras expression promotes the release of exosomal TGF-β. Exosomes secreted by 21D1 cells (Ras-transformed MDCK cells) induced higher TGF-β signalling activity as compared with exosomes from MDCK cells. In particular, Ras expression resulted in the enrichment of active TGF-β in exosomes as well as a significant increase in exosome production (Fig. 5.1). This was further supported by the observation that exosomal TGF-β levels were higher in Ras-transformed NIH3T3 cells than NIH3T3 cells (Fig. 5.2). Also, enhanced secretion of exosomal TGF-β was observed when HEK 293T cells were transiently transfected with v-Ha-Ras encoding plasmids (Fig. 5.3). Collectively, our data suggests a key role played by Ras in the production of exosomal TGF-β.

It is well recognized that *RAS* (*HRAS*, *KRAS* and *NRAS*) are the most frequently mutated oncogenes in human cancers [233]. Among the three Ras isoforms, K-Ras mutations are found in 30% human cancers [264] and comprise the highest percentage (86%) of all Ras mutations [235], while H-Ras has been most extensively studied. Oncogenic H-Ras-induced transformation has been shown to alter the protein expression in NIH3T3 fibroblasts [261]. Also, Ras-transformation in MDCK cells causes global changes in exosome proteome by up-regulating mesenchymal markers and proteinases [119]. In the present study, we uncover a role of H-Ras as a crucial player in the secretion of exosomal TGF-β. Because K-Ras and N-Ras are more frequently mutated in human cancers (30% and 8%, respectively [233]), it will be interesting to determine in the future studies if K-Ras and N-Ras can also mediate the production of exosomal TGF-β.

Ras proteins appear as pivotal signalling transducers that modulate multiple biological processes, including proliferation, invasion and survival. Ras proteins have been shown to recruit and activate PI3K in endosome [36], which contributes to MVBs formation and exosome biogenesis [37, 38]. In addition, we found by immunoblotting that Rab27a, a Ras-related protein that controls exosome secretion and the levels of exosomal TGF-β (Chapter 3, Fig. 1F), was markedly increased upon Ras expression (data not shown). As such, Ras-mediated up-regulation of exosome release (Fig. 5.1-5.3 E) is likely to be PI3K- or Rab27a-dependent.

Interestingly, our results demonstrated that Ras expression resulted in the enrichment of active TGF-β in exosomes (Fig. 5.1-5.3D). It has been shown that Ras signalling synergizes

with TGF-β signalling in EMT by promoting autocrine secretion of TGF-β, which undergo endocytosis and are targeted to endosomal trafficking in the presence of TβRI and TβRII [95, 148]. Because exosomes are generated from endosomes, Ras-mediated enrichment of active TGF-β in exosomes may occur through a mechanism which involves Ras-induced autocrine loop of TGF-β.

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